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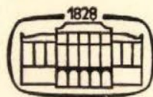
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TOMUS XXIII

FASCICULUS I



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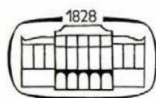
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TOMUS XXIII

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PATHOGENITÄTSNACHWEIS DER STAPHYLOKOKKEN MIT ADRENALIN

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(Eingegangen am 18. April 1974)

M. SELLI (Cekmece Nuclear Research Center, Laboratory of Microbiology, Istanbul and Department of Bacteriology and Serology, Clinic Steglitz FU Berlin, Berlin): **Pathogenicity test for staphylococci by the use of adrenaline.** Pathogenic staphylococci will grow in broth containing 50 µg/ml adrenaline at 37 °C, while apathogenic ones tolerate a concentration of 20 µg/ml only. They are completely inhibited at higher adrenaline levels. After 6 hr incubation, pathogenic germs show growth up to 100–250 µg/ml adrenaline, apathogenic ones up to 40 µg/ml.

Zusammenfassung. Eine Methode zur Differenzierung von pathogenen und apathogenen Staphylokokken binnen 3–4 Stunden wurde ausgearbeitet. Während sich in Bouillon mit, 50 µg/ml Adrenalinhalt pathogene Staphylokokken noch gut vermehren, vertragen apathogene Staphylokokken nur eine Adrenalinkonzentration von 20 µg/ml. Nach 6stündiger Bebrütung wuchsen pathogene Staphylokokken bei 100–250 µg/ml, apathogene Staphylokokken jedoch nur bei 40 µg/ml Adrenalinhalt.

Zur Differenzierung von pathogenen Staphylokokken wird in Routine-laboratorien vor allem die Plasmacoagulase-Reaktion verwendet. Staphylokokken, die das Plasma koagulieren, werden als pathogen angesehen und als *Staphylococcus aureus* bezeichnet. Staphylokokken, die Plasma nicht koagulieren, werden als apathogen betrachtet und als *Staphylococcus epidermidis* bezeichnet. Daneben wird auch Schnellagglutinationstest (Objektträgermethode) in vielen Laboratorien verwendet. Hierbei wird der an die Keime gebundene Clumping-Factor nachgewiesen. In positiven Fällen tritt oft schon während der Mischung von Plasma und Kolonie eine Verklumpung infolge Fibrinpräzipitation an der Oberfläche der Keime auf [1]. Diese schnelle Methode ist auch zuverlässig. Doch in stark beschäftigten Laboratorien wird nicht einmal Plasmacoagulase-Reaktion durchgeführt, da die Zeit fehlt, 1–2 Tage auf die Ergebnisse zu warten, und *S. aureus* wird einfach auf Grund der Pigmentbildung oder β -Hämolyse diagnostiziert.

Es wurde mehrmals nachgewiesen, daß manche Stämme, die das Plasma nicht koagulieren und nicht agglutinieren, doch pathogen sind. PULVERER und HALSWICK [2] fanden 34, und KASIMOGLU [3] 7 *S. epidermidis* Stämme, die sich als Krankheitserreger erwiesen.

Zum Pathogenitätsnachweis der Staphylokokken wurde zuerst Leukozydin verwendet [4]. Später wurde diese Eigenschaft auf Grund der Mannitspal-

tung [5], Lysozymbildung [6, 8], DNase [9], Carotinolysin [10], Enterotoxin [11–13], β -Hämolyse [14], Hyaluronidase [15], Staphylocoagulase [16–18], Fibrinolysin [19, 20], Collagenase [21] u. a. beurteilt. Wir können leider nicht annehmen, daß alle pathogenen Stämme alle diese Eigenschaften besitzen. Manche Stämme zeigen keine β -Hämolyse, aber Bildung von DNase, Lysozym und Coagulase; in einem anderen Stamm läßt sich Lysozym, aber keine Plasmacoagulase und DNase nachweisen. Bis jetzt wurde keine Eigenschaft gefunden, die bei allen pathogenen Staphylokokken vorhanden und unbedingt charakteristisch wäre.

Material und Methode

Die untersuchten Stämme von *S. aureus* und *S. epidermidis* wurden von Kliniken erhalten, wo die aureus-Stämme als Krankheitserreger beurteilt wurden. Alle diese Stämme wurden in unserem Laboratorium kontrolliert, und für Plasmacoagulase, DNase, β -Hämolyse, Pigmentbildung, Plasmaagglutination und Lysozymbildung positiv gefunden. Die *S. epidermidis* Stämme besaßen diese Eigenschaften nicht.

Als Nährboden diente gewöhnliche Bouillon. In der L-Adrenalin in 1, 2, 3, 3.5, 5, 7, 10, 15, 20, 25, 30, 40, 50, 60, 75, 100, 125, 150, 200, 250, 300, 350, 400 $\mu\text{g/ml}$ Konzentration zugesetzt wurde. Nach 24 St Inkubation in gewöhnlicher Bouillon wurden jeweils 10^4 Keime in ein Adrenalin-Bouillon enthaltendes Röhrchen beimpft und bei 37°C weiter bebrütet. Das Ablesen des Wachstums erfolgte jeweils nach 1, 2, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 10, 12, 24 Stunden.

Ergebnisse

Alle *S. aureus*-Stämme wuchsen in den ersten 3–3.5 Stunden bis 50 $\mu\text{g/ml}$ Adrenalinkonzentration, während die meisten *S. epidermidis*-Stämme nur 15 $\mu\text{g/ml}$ Adrenalin vertrugen, einzelne Stämme wiesen bei 20 $\mu\text{g/ml}$ Adrenalin noch geringes Wachstum auf; durch höhere Konzentrationen waren die letzteren völlig gehemmt. Nach 4–5stündiger Bebrütung bei gleichen Bedingungen zeigte sich Wachstum bei pathogenen Stämmen bis 75 $\mu\text{g/ml}$ bzw. 100 $\mu\text{g/ml}$, bei den apathogenen Stämmen nur bis 20 $\mu\text{g/ml}$ bzw. 25 $\mu\text{g/ml}$ Adrenalin. Nach 6stündiger Bebrütung wuchsen bei gleichen Bedingungen manche *S. aureus*-Stämme von 100 $\mu\text{g/ml}$ bis 250 $\mu\text{g/ml}$, während *S. epidermidis*-Stämme nur 40 $\mu\text{g/ml}$ ertrugen (s. Tab. I). Das wichtigste Kriterium war die Periode zwischen der 3. und 4. Stunde. In diesem Zeitpunkt konnten *S. epidermidis* und *S. aureus*, oder pathogene und apathogene Staphylokokken genau differenziert werden.

Aus den 96 getesteten pathogenen Staphylokokkus-Stämmen waren bei 80 Stämmen die Plasmakoagulase, DNase, β -Hämolysin, Plasmaagglutination und Pigmentbildung positiv; bei 16 Stämmen fanden wir nicht alle diese Eigenschaften (Tab. II). Manche waren Plasmakoagulase positiv, manche Plasmakoagulase negativ, oder es fehlten andere Charakteristika. Sie wuchsen genau wie andere *S. aureus*-Stämme. Wir kontrollierten gleichzeitig 75 *S. epi-*

Tabelle I

Wachstum von Staphylokokken nach 3.5 Stunden Bebrütung

Stämme	$\mu\text{g/ml}$ Adrenalin					
	10–20	25	30	40	50	60–400
<i>S. aureus</i> , 80 Stämme	+	+	+	+	+	—
<i>Staphylococcus</i> *						
Nr. 119, 157, 283, 321, 366, 478, 491, 849, 850, 874	+	+	+	+	+	—
Nr. 166, 384, 386	+	+	+	(+)	(+)	—
Nr. 170, 171, 698	+	+	+	+	(+)	—
<i>S. epidermidis</i> , 75 Stämme	+	—	—	—	—	—
<i>S. epidermidis</i> *						
Nr. 34, 875, 655, 670	+	+	+	+	+	—
Nr. 19, 232, 653, 654	+	+	+	(+)	(+)	—

* Als Krankheitserreger aus Patienten isolierte Stämme

dermidis-Stämme, die keine solche Charakteristika besaßen. In der Albus-Gruppe wurden 8 andere Stämme mitgetestet. Von diesen hatten No. 19 und 34 nach dem 3. Tag bei Zimmertemperatur leicht Plasma koaguliert und Nr. 232, 653, 670, 875 bildeten in dieser Zeit einen leichten gelblichen Farbton. Die anderen 2 Stämme Nr 654 und 655 besaßen diese Eigenschaften nicht. Diese 8 Stämme sind von Patienten als Krankheitserreger isoliert worden. Sie wuchsen auch bei den hohen Adrenalin-Konzentrationen wie *S. aureus*. Unmittelbar von Platten gewonnene Staphylokokken zeigten ein ähnliches Wachstum in Bouillon mit Adrenalin wie Keime, die vorher 24 Stunden lang in Bouillon gezüchtet wurden.

Besprechung

Unsere Ergebnisse zeigten, daß bei hohen Adrenalin-Konzentrationen *S. epidermidis*-Stämme nach einer Zeit kein Wachstum mehr aufweisen, dagegen vermehren sich *S. aureus*-Stämme, die Krankheitserreger sind, auch bei hohen Adrenalin-Konzentrationen. Die anderen 16 Stämme, die nicht die gleichen Eigenschaften wie normale *S. aureus*-Stämme zeigten und auch als Krankheitserreger isoliert waren, wuchsen genauso gut bei hohen Adrenalin-Konzentrationen wie *S. aureus*-Stämme. Die 8 anderen *S. epidermidis*-Stämme, die auch als Krankheitserreger isoliert wurden, wuchsen ähnlich wie die pathogenen Staphylokokken. Im Schrifttum ist über Hormonwirkungen auf das Wachstum der Mikroorganismen nicht viel zu finden. CAPEK *et. al.* [23] fanden, daß Steroidhormon in hohen Dosen eine Inhibition bewirkt. YOTIS and

Tabelle II

Charakteristika der Staphylokokken

Stämme	Stämme Nr.	DNAse	Plasma- koagulase	Plasma- agglutina- tion	β-Hämolyse	Lysozym	Pigment- bildung
<i>S. aureus</i> , 80 Stämme		+	+	+	+	+	+
<i>Staphylococcus</i> *	119	+	+	+	+	—	—
	157	+	—	—	(+)	+	(+)
	166	(+)	—	—	—	(+)	+
	170	+	—	—	(+)	+	+
	171	+	—	—	(+)	+	+
	283	(+)	+	+	+	+	+
	321	(+)	+	+	+	—	+
	366	(+)	(+)	+	+	(+)	+
	384	—	—	—	+	+	—
	386	+	—	—	+	—	—
	478	+	+	+	+	—	(+)
	491	+	+	+	—	—	—
	498	—	+	+	+	—	—
	849	+	+	+	+	(+)	+
	850	—	+	+	+	+	(+)
	874	+	+	+	+	—	(+)
<i>S. epidermidis</i> , 75 Stämme	—	—	—	—	—	—	—
<i>S. epidermidis</i> *	19	—	(+) ³	—	—	—	—
	34	—	(+) ³	—	—	—	—
	232	—	—	—	—	—	(+) ³
	653	—	—	—	—	—	(+) ³
	654	—	—	—	—	—	—
	655	—	—	—	—	—	—
	670	—	—	—	—	—	(+) ³
	875	—	—	—	—	—	(+) ³

* Als Krankheitserreger aus Patienten isolierte Stämme

+ = positiv

(+) = leicht positiv

(+)³ = leicht positiv, 3. Tag

— = negativ

FITZGERALD [24] injizierten pathogene Staphylokokken in Ratten und behandelten sie mit Androgenen. Die unbehandelten Tiere erkrankten, während bei den Behandelten eine antimikrobielle Wirkung beobachtet wurde.

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THE MACROCONIDIAL SEPTUM OF SOME DERMATOPHYTON SPECIES

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Summary. The septum of the macroconidium (MC) of dermatophytes was examined by light microscopy. Significant taxonomical differences between species have not been found. The septa seem to be three-layered, but only the middle layer can be considered a septum in the wall substance of two neighbouring chambers, impressed and completed by the septum. The septum verum originates from the middle layer of the three-layered MC wall or from its surface adjacent to the chamber wall. This type of septum is different from that described in *Ascomycetes* and other species. Light microscopic findings are insufficient to characterize the nature of the septal pore. The microscopic observation of the layers of the MC wall and of the septum may be disturbed by misleading optical phenomena.

The macroconidium (MC) (closterospore, fuseau, macroaleuriospore) appearing in cultures of dermatophytes may be characterized as follows. It is an immobile, (as compared with hyphae) swollen multicelled (multiseptate) formation sitting on the apex, or on a sterigma at the side, of fertile hyphae, single or in clusters, sometimes ramifying from one another. Its wall, slightly or much thicker than the hyphae, may show surface ornamentation (warts, tubercles).

According to the "Dictionary of the Fungi" [1] "macroconidium (is) (i) the longer, generally more diagnostic, conidium of a fungus which has microconidia in addition; (ii, infrequent) a long or large conidium".

Obviously, the MC of the *Microsporum* and *Trichophyton* species is covered by definition (i), whereas those of the *Epidermophyton* species including *E. floccosum*, *E. ajelloi*, *E. longifusum* are covered by definition (ii).

The physiological role of the MC is unclear; its taxonomical role is of more importance. Among the light microscopic properties of the MC, the following have been utilized in classification: its presence or absence, its location, shape and size, the number of its chambers, the thickness of its wall, and the ornamentation of its external surface [2].

In contrast, the properties of the walls separating the chambers of the MC have been neglected. Only CAPRILLI *et al.* [3] have discussed this question, without finding much response.

In the routine diagnostics of dermatophytes, fungi usually appearing in imperfect form, the features observable by light microscopy will remain in the focus of interest for a considerable time. Classification on serological, chemo-

taxonomic or physiological basis is time-consuming, while the dynamic morphological observations of conidiogenesis, widely used recently in the taxonomy of *Deuteromycetes* [4-8] is still in the initial stage as regards the differentiation of dermatophytes [7,9].

The septum of the MC is easy to observe in stained as well as unstained preparations. It is therefore of interest to show whether it is a homologous or an analogous organ in the different dermatophyte species and whether it is of taxonomic importance within this groups of fungi.

Materials and methods

The samples for microscopic observation were taken from Sabouraud glucose agar cultures showing well-defined fructification (12-18-day cultures). Conventional lactophenol-cotton blue preparations were studied by normal light microscopy and by phase-contrast microscopy. Further methodical data and the experimental models are presented in the Figures.

Results and discussion

Septum in general means a cell wall or a separating wall. TALBOT [10] differentiated primary septa and accessory septa. The primary septum separates the daughter cells from each other after mitosis or meiosis; it has a pore, which may develop into a doliporus in *Basidiomycetes* and may be in connection with Woronin's bodies in *Ascomycetes*. The accessory septum is formed in the absence, or independently of, nuclear division, most frequently associated to a poorly known process, viz., the migration of the cytoplasm from one part of the cell into another. The identity or equivalence of MC septa to the septa of hyphae has not been proved.

Further discussion will be restricted to the genera *Microsporum* and *Epidermophyton*, considering that the walls and the septa of the MC of *Trichophyton* species are difficult to investigate. They are much thinner and hardly separated from the hyphae and not more differentiated than the latter. We assume that they tend to become rudimentary.

In the genera *Microsporum* and *Epidermophyton* the MC chambers separated by septa are equivalent to cells. Each is capable of germination, whether split into chambers or having remained in the intact MC. Young septa allow some communication between chambers, viz. the cotton blue dye dissolved in lactophenol, diffuses into the middle chamber from the apex or from the base. In older MC the septum is less permeable, the dye does not penetrate across the apex, and the dye having penetrated at the base takes several weeks to reach the second chamber. The tendency to splitting of the MC observed in several species suggests that the septum consists of two or three layers. Microscopically, the septa of the intact MC show two hyaline layers and a darker layer

between them. In spite of its darker appearance, the middle layer does not take up dye; it thickens towards the periphery and slightly extends into the main wall. Each hyaline layer is part of a layer lining the wall of a chamber. Owing to its hyaline nature, it needs staining of the chamber's contents to be well distinguished (Fig. 1). It cannot be excluded that still more layers could be differentiated by electron microscopy.

From the early (swelling) phase of macroconidiogenesis, the MC wall, though thin, consists of three layers. It is covered by a strongly light-absorbing layer (outer layer in Fig. 1). In the genus *Microsporum* the ornamentation of the

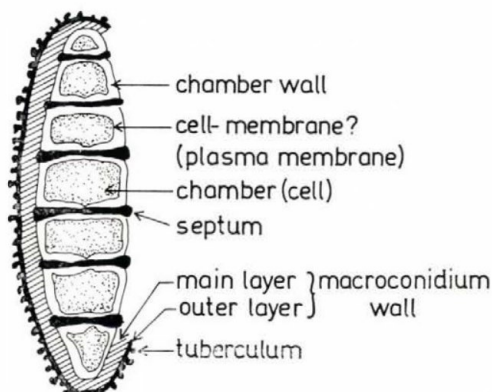


Fig. 1. Schematic drawing of an MC of the *Microsporum* type

surface develops from this layer. The main layer lies beneath the outer layer. It is also thin in the initial phase, but grows in thickness especially intensely in certain *Microsporum* species. This layer is much more translucent than the outer one. It stains slightly, if at all, with lactophenol-cotton blue. Septa may develop from this layer or from unknown foci adjacent to its surface. The initial, peripheral part of the centripetally growing septa appears to be embedded in this layer. The third, innermost layer of the MC wall (inner layer in Fig. 1) remains transparent after lactophenol-cotton blue staining. At the initial phase of septum formation this layer is pressed inwards, for the septa begin their centripetal ring-like growth from beneath this layer. This process can be followed by light microscopy until the septum is complete and thus two or more separate septa are formed. In this way the inner layer appears to form a continuous chamber wall covering the whole cytoplasm. Though invisible under the light microscope, it is reasonable to suppose on a cytological basis that a cell membrane exists between the cytoplasm and the chamber wall. Accordingly, only the formation described above as three-layered, and separating two chambers from each other can be considered a septum, *i.e.*, a new

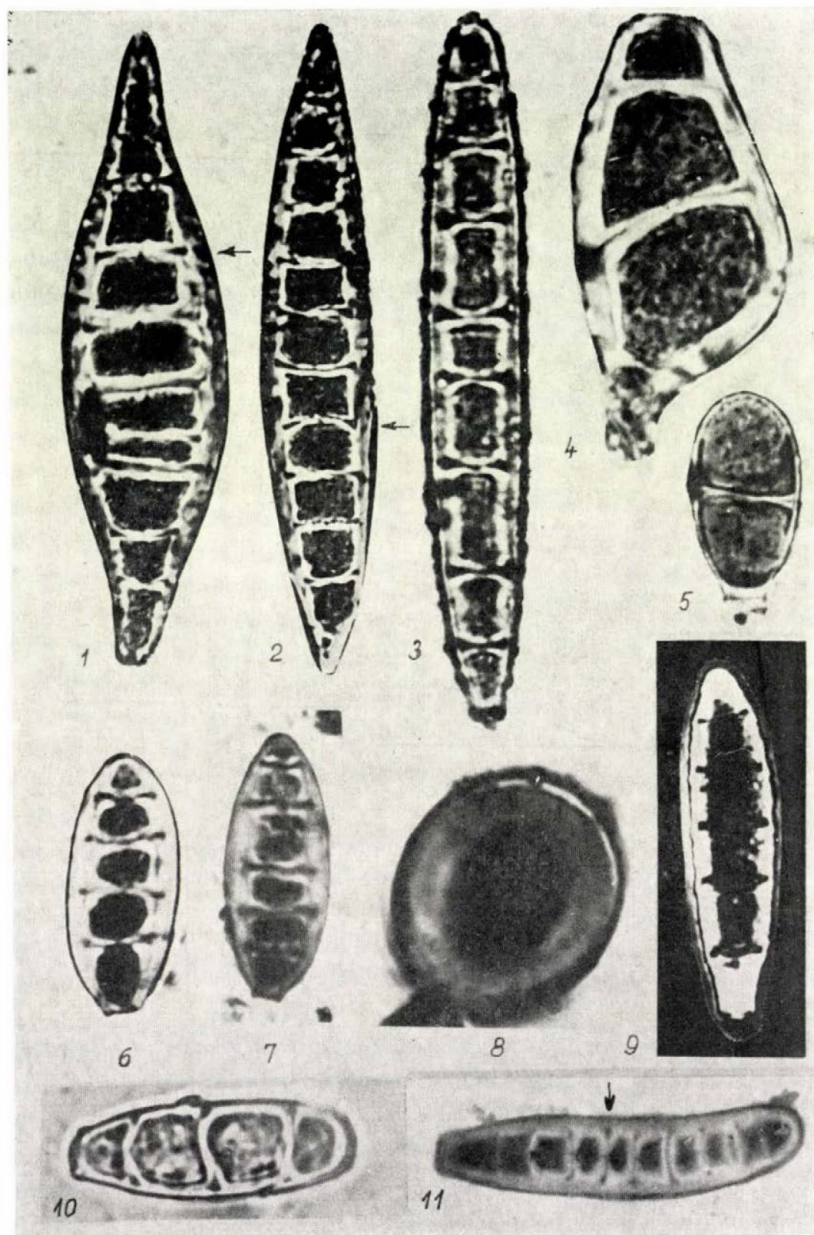


Fig. 2. Macroconidia of *Microsporium* and *Epidermophyton* species. Lactophenol-cotton blue preparations. 1-2. *M. felineum* (original magnification, $\times 160$); 3. *M. vanbreuseghemii* ($\times 160$); 4. *M. umbonatum* ($\times 380$); 5. *M. nanum* ($\times 380$); 6-7. *M. cookei* ($\times 250$); 8. *M. cookei*, from the direction of the longitudinal axis ($\times 570$); 9. *M. gypseum*, phase contrast ($\times 250$); 10. *E. floccosum* ($\times 250$); 11. *E. ajelloi* ($\times 250$). Arrows point to the dark triangles at the junction of the septum and the MC wall

formation in the strict sense. This layer exceeds in light absorption not only the chamber wall, but also the main layer of the MC wall. This, however, does not yet prove that the septum is different from the main layer of the wall in origin or in structure. For the seemingly more intensive light absorption an increased thickness due to concurrence of the plane of section and of the axis of observation might be responsible. As to the shape of the septum we can not agree with the conception of CAPRILLI *et al.* [3]. In our opinion the septum resembles a biconcave disk, therefore its plane of section appears as two triangles touching each other at their acute angles, or in the shape of a propeller (Fig. 2).

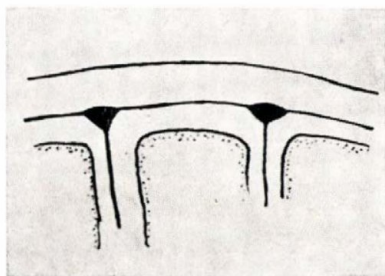


Fig. 3. Schematic drawing of the triangular formation described by CAPRILLI *et al.* [3] at the "septum membrane" - "MC-wall membrane" junction

Unlike CAPRILLI *et al.* [3], we only found a difference in the thickness of the MC wall and in the thickness of its layers in the species *M. cookei*, *M. felineum* (syn. *M. canis*), *M. gypseum*, *M. nanum*, *M. racemosum*, *M. umbonatum* (syn. *M. distortum*) [11] and *M. vanbreuseghemii*. We found no structural differences even in incomplete septa (Fig. 3).

Furthermore, CAPRILLI *et al.* [3] regard as a membrane the thin linear formation running parallel with the MC wall and suggest that the transversal membrane is attached to a swelling of this formation. This might be rather an optical phenomenon reflecting the limiting surface between the main layer of the MC and the chamber wall. Were it a cytoplasmic membrane, it would be located between the cytoplasm and the cell wall. The formation described by the authors quoted as a transversal membrane is regarded by us as the septum. Its substance seems to resemble a wall rather than a membrane, also in *M. cookei* and *M. vanbreuseghemii*, and a membrane should be located between the cytoplasm and the hyaline wall of the chamber. As regards the bilateral swelling at the junction of the "membrane" inside the MC wall and the "transversal membrane", if anything is visible there, it is not two structures but a single formation: the optical appearance of a ring-like organ on both sides of the plan of observation. In addition, the structure observed by us in that region does

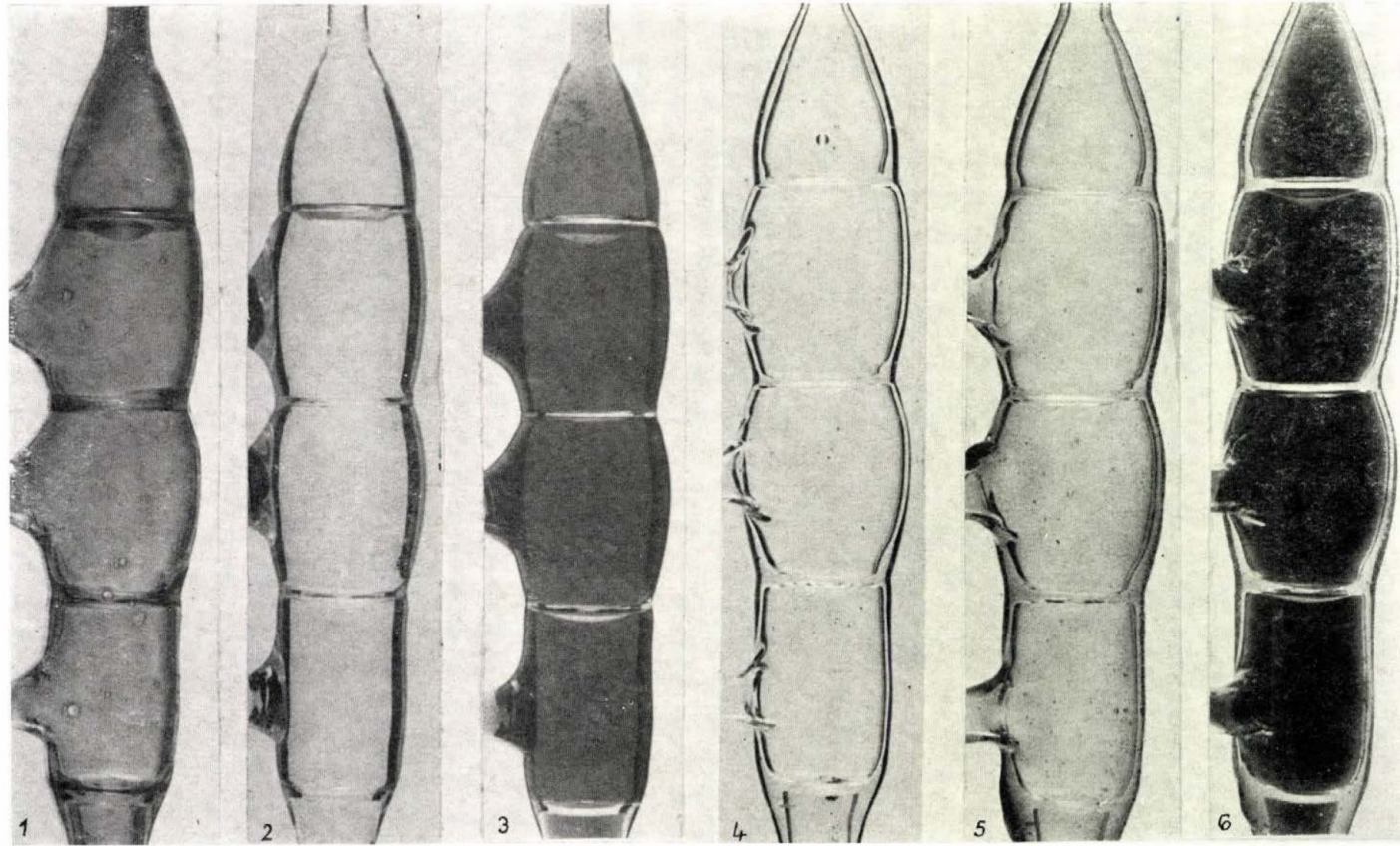


Fig. 4. Glass MC model 1. outer surface varnished, empty; 2. outer surface unvarnished, filled with water; 3. unvarnished, filled with methylene blue solution; 4. unvarnished, filled with water; 5. outer surface varnished, filled with water; 6. outer surface varnished, filled with methylene blue solution. (1-3, in air; 4-6, immersed in water)

not resemble an equilateral triangle, its angle pointing inwards being much more acute; it corresponds to the optical image of the biconcave septal disk.

As regards the "septal pore", it is difficult to form an opinion concerning its nature on the basis of its light microscopical appearance. The existence, development and structure of the pore cannot be separated from the development of the septum, and the MC septum of dermatophytes cannot be adapted to MOORE's [12] classification. This is not surprising since this classification is based on other organs (hyphae) and the dermatophyte MC is different from other macroconidia in that its septum is covered by a chamber wall on both sides.

The septum, whether or not it has a pore, is permeable in the early stage, but impermeable later on. Furthermore, a sudden parallel thinning of both the septum and the chamber wall cannot be detected in every septum of the MC (Fig. 2, MC 1, 2, 4 and 6).

The possibility of an optical error is illustrated in Fig. 4 by serial photos of a model. The model consisted of a single glass layer or of two layers, glass and varnish. The model was illuminated by dispersed light. In Picture 4, the one-layered wall appears as three-layered; in Picture 6, the wall seems to be composed of four layers. In this latter picture, the innermost transparent layer imitates well that designated above as chamber wall. In several pictures, especially in Picture 3, the MC wall seems to be thicker than it was in reality.

At the septum-MC wall junction a characteristic triangular form is visible in several pictures, especially in Pictures 1 and 2. The septum seems to be multilayered in Picture 3 and a septal pore is apparent in Pictures 1, 3 and 5.

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INTERFERON PRODUCTION BY HUMAN LEUKAEMIC LEUCOCYTES

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Summary. The Sendai virus-induced interferon (IF) production by partially purified human leucocyte suspensions of normal donors and of leukaemic patients have been investigated *in vitro*. (i) An increased production was observed with leucocytes taken from patients suffering from chronic myelogenous leukaemia (CML) during exacerbation, but the production was approximately normal with cells taken during remission. (ii) IF production was not influenced by large doses of cytostatics (DBM, 5-FU, FUDR, 5-HU, 6-MP, cyclophosphamide) irrespective of whether normal or CML leucocytes were used. (iii) In contrast, production was inhibited in both systems by inhibitors of RNA or protein synthesis (actinomycin D, puromycin, cycloheximide). (iv) CML leucocytes produced IF for a prolonged period of time as compared to normal leucocytes. (v) Leucocytes from children with acute blastic leukaemia and those from adults with chronic lymphoid or acute paramyeloblastic leukaemia produce, in contrast to normal leukocytes, approximately as much IF in the absence as in the presence of serum. It is concluded that the Sendai virus-induced IF synthesis in CML leucocytes requires *de novo* protein synthesis.

GRESSER [1] was the first to report on virus-induced interferon (IF) production *in vitro* by human leucocytes. Since then the phenomenon has been studied by numerous authors [2-16].

It has been shown that human leucocytes from healthy donors, when incubated *in vitro* and induced with Sendai virus, produce much less IF in the absence than in the presence of serum [4, 7, 10, 12]. Serum albumin, cow's milk or milk casein combined with a dipolar-ionic buffer can be substituted for the serum [17, 18].

IF production *in vitro* by leucocytes from patients with chronic lymphoid leukaemia (CLL) is lower, whereas that by chronic myeloid leukaemic (CML) leucocytes is higher, than the production by normal leucocytes [19-25].

In the present report, (i) results of our investigation on the IF production of CML leucocytes are summarized, with special regard to the stages of the illness and the applied cytostatic therapy; (ii) the results of IF production *in vitro* by leucocytes from five patients with paramyeloblastic leukaemia are described (iii) and also those concerned with the influence of cytostatics on the IF production *in vitro* by CML leucocytes and (iv) with the possible mechanism of IF production by leukaemic leucocytes.

Materials and methods

Chemicals. Actinomycin D (Merck, Sharp and Dohme, Ltd.). Puromycin dihydrochloride (Nutritional Biochemicals Co., Cleveland). Cycloheximide (Acti-Dione, Calbiochem, Los Angeles). Dibrommannitol (Myelobromol, Chinoïn, Budapest). 5-FU: 5-fluorouracil (Serva, Heidelberg). FUDR: 5-fluoro, 2'-deoxyuridine (Serva, Heidelberg). 5-HU: 5-hydroxyurea (Sigma). 6-MP: 6-mercaptopurine (Wellcome Research Laboratories). Cyclophosphamide (Endoxan, Wellcome Research Laboratories).

Viruses. The Sendai strain of parainfluenza virus type 1 was maintained in embryonated hen's eggs by serial allantoic passages. The virus-containing allantoic fluids were kept at +4°C until used. The haemagglutination (HA) titres were determined by TAKÁTSY's [26] Microtiter technique. The Indiana strain of vesicular stomatitis virus (VSV) was propagated in cultures of the U-line of human amniotic cells. The virus was stored at -70°C.

Cell cultures. U-line cells were cultured in Parker's medium 199 containing 10% inactivated calf serum and 0.125% sodium hydrocarbonate.

Purification of leucocytes and interferon production. These procedures were performed as described by STRANDER and CANTELL [6, 7]. Purified leucocytes (if not indicated otherwise in the text) were suspended in medium 199 of pH 7.2, enriched with 10% calf serum; the cell count was adjusted to 5×10^6 cells/ml, and the suspensions, distributed in siliconized centrifuge tubes, were inoculated with Sendai virus (100 HA units/ml). The tubes were then stopped tightly and incubated in a roller drum at 37°C for 24 hr (or for different periods of time shown in Fig. 1).

Interferon was determined as described earlier, in tube cultures of U-line cells. For this purpose a fourfold dilution series of each preparation was tested for inhibition of the cytopathogenic effect of VSV. The highest dilution protecting 50% of the cultures from the pathogenic effect of the virus was accepted as IF titre [20].

Blood donors. The patients were thoroughly examined at the Departments of Medicine and Paediatrics, University Medical School, Debrecen. The stage of the illness (exacerbation or remission) was registered. It was also registered whether the patient was under cytostatic therapy at the time of blood sampling. Blood samples from healthy controls were kindly supplied by the Department of Blood Supply, County Hospital, Debrecen, or, occasionally, from patients free of haematological, immunological and infectious disease.

The patients' leucocytes and the corresponding control leucocytes were taken at the same time and examined parallel with exactly the same technique.

Results

IF production by CML leucocytes. Blood was taken from 22 adult patients, *viz.*, one sample each from 6 and several samples from the others. A total of 75 samples were tested. Of these, 50 had been taken during exacerbation and 25 during remission.

Interferon production *in vitro* by the CML leucocytes taken during exacerbation was significantly higher than production by the control leucocytes. The geometric mean for the IF titres was 584 and 66, respectively. The significance of this difference was at the 0.01 level. On the other hand, there was no appreciable difference in this respect between leucocytes taken during remission and the control leucocytes. The respective geometric means were 57 and 61.

In Table I the IF production by leucocytes obtained from consecutive blood samples of five patients are shown. The patient/control quotients of IF titres were higher in each case with leucocytes taken during exacerbation than with those taken during remission.

Table I

IF production in vitro by leucocytes from patients with CML and by those from healthy controls

Patients' initials	Blood sample No.	Phase of illness*	Cytostatic therapy**	IF titres	
				patient/control	quotient
T. L.	1	R	No	24/32	<1
	2	R	No	16/32	<1
	3	R	DBM	128/64	2
	4	E	No	512/32	16
	5	E	DBM+M	2048/64	32
	6	R	M	512/256	2
	7	E	DBM+M	512/64	8
J. J.	1	E	No	256/32	8
	2	R	No	16/16	1
	3	E	DBM	256/16	16
	4	E	DBM	8192/512	16
	5	E	DBM	16000/256	64
	6	R	No	64/64	1
	7	E	Z+Pr.	8192/512	16
L. P.	1	E	Z	256/4	64
	2	R	DBM	16/512	<1
L. M.	1	E	No	3200/128	25
	2	E	DBM	4096/32	128
	3	R	DBM	64/128	<1
	4	R	Z	128/128	1
T. S.	1	E	No	256/16	16
	2	R	DBM	16/16	1
	3	E	Z	2048/256	8

* E: Stage of exacerbation; R: stage of remission

** DBM: Myelobromol; M: busulfan; Pr.: prednisone; Z: mannosulfan

IF production by leucocytes taken from patients with acute myeloblastic leukaemia. Leucocytes from five adult patients were examined. IF production was elevated in three cases, reduced in one case and approximately equal to the control in one case.

Effect of immunosuppressive (cytostatic) drugs on IF production in vitro by leucocytes from healthy donors and those from patients with CML. The cytostatics were used in the following concentrations. DBM: 50 and 25 µg/ml; 5-FU: 200 and 100 µg/ml; FUDR: 200 and 100 µl/ml; 5-HU: 100 and 50 µg/ml; 6-MP: 100 and 50 µg/ml; cyclophosphamide: 200 and 100 µg/ml.

Leucocytes from 7 patients and control leucocytes were used. The freshly prepared solution of the cytostatics and the Sendai virus were added to the cells simultaneously and the cells were incubated with the drug for 24 hr after inoculation. IF production was not influenced by any of the drugs, irrespective of the origin of the leucocytes.

Experimental approach to the mechanism of IF production by leukaemic leucocytes.

(a) Metabolic inhibitors (actinomycin D, puromycin, cycloheximide), and leucocytes taken from patients during exacerbation of CML were used in these experiments. The antimetabolites and the Sendai virus were added to the cell suspension simultaneously and the cells were incubated with the antimetabolite for 24 hr after inoculation (Table II).

Table II

Effect of antimetabolites on IF production by leucocytes obtained from CML patients and healthy donors

Initials of patients	Actinomycin D ($\mu\text{g/ml}$)	Puromycin ($\mu\text{g/ml}$)	Cycloheximide ($\mu\text{g/ml}$)	IF titre	
				Patients	Controls
T. L.	—	—	—	512	32
	2	—	—	<4	<4
	—	—	—	4096	1024
T. I.	—	10	—	<4	<4
	—	—	10	<4	<4

IF production was prevented by each antimetabolite, irrespective of the origin of the leucocytes.

(b) In several experiments the course of IF production was determined, using leucocytes taken from CML patients during exacerbation and control leucocytes.

The curves in Fig. 1 show that the control leucocytes stopped producing IF 8–9 hr after infection, whereas the CML leucocytes produced in the period 8–24 hr after inoculation 8 to 32 times the same amount of IF that had been produced in the first 8 hr. In the first 8 hr the difference in IF production between CML and control cells was inconsistent.

(c) IF production by leucocytes was comparatively examined in the presence and in the absence of serum. The leucocytes originated from patients with different forms of leukaemia and from healthy donors. Serum had not been used during the procedure of leucocyte purification (Table III). The leucocytes from patients with blastic leukaemia and the CLL leucocytes produced nearly as much IF in the absence as in the presence of serum.

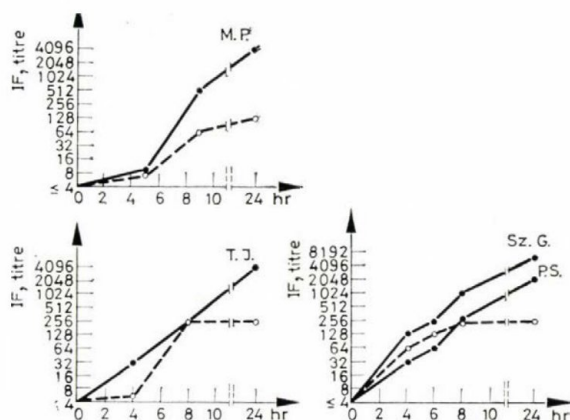


Fig. 1. IF production by leucocytes from patients with CML and by those from healthy controls.
 ●—● CML cells; ○—○ normal cells. Three experiments

Table III

Interferon production by leucocytes from patients with various forms of leukaemia and from healthy donors in the presence or absence of serum

Initials	Age, year	Patients		Serum per cent	IF titre	
		Disease	Blood sample No.		Patients	Controls
K. J.	8	Acute blast-cell leukaemia	277	10 0	4000 2000	128 8
K. J.	8	Acute blast-cell leukaemia	284	10 0	4000 2000	128 4
K. J.	8	Acute blast-cell leukaemia	286	10 0	1000 1000	128 16
K. J.	3	Acute blast-cell leukaemia	297	10 0	512 512	128 4
M. J.	26	Acute paramyeloblastic leukaemia	257	10 0	512 256	32 <4
B. B.	39	CML	262	10 0	8000 <4	512 <4
T. J.	37	CML	276	10 0	2000 128	512 8
B. G.	53	CML	306	10 0	512 32	128 4
H. J.	55	CLL	267	10 0	512 512	512 8
B. G.	66	CLL	340	10 0	64 64	256 16

Discussion

The present results have confirmed on a relatively large clinical material the results published by other investigators [21–23] and ourselves [20], that the virus-induced IF production by CML leucocytes *in vitro* may be of increased intensity. In addition, we have shown that the increased IF production depends on the stage of CML at which the leucocytes were taken (Table I).

IF production by leucocytes obtained from adult patients with acute paraneoplastic leukaemia showed a wide variation. Other investigators obtained low IF production by leucocytes from adult patients with ALL [19] and normal production by leucocytes from children suffering from blastic leukaemia [24].

Patients under immunosuppressive treatment were found to have an increased susceptibility to viruses [27, 28], first of all to those of the herpesvirus group, and the consequent complications may aggravate the conditions of the patient (*e.g.*, after renal transplantation) [29–31]. Furthermore, the incidence of tumours (lymphomas and reticulocytic sarcomas in the first place) was raised in patients under immunosuppressive treatment after renal transplantation [32–36]. In this phenomenon, oncogenic viruses have been supposed to play a part [37, 38]. It is therefore of interest to know whether IF production, an important factor of the antiviral and perhaps antitumorigenic defence mechanism, is influenced by the immunosuppressive therapy. It has recently been reported that the human cytomegalovirus can transform cells *in vitro* [39] and that the IF production (inducible by NDV) by the leucocytes of children with congenital cytomegalovirus infection was poor [40].

According to the present results, the Sendai virus-induced IF production by either normal or CML leucocytes cannot be reduced by certain cytostatics alone even when these are used at concentrations higher than therapeutical. According to another report [41], the NDV-inducible IF production *in vitro* by leucocytes from kidney-transplanted patients under prolonged corticosteroid and azathioprin treatment was lower than the control production.

We have confirmed our own earlier results [12, 20] and those of LEE [42] indicating that the IF production by normal and CML leucocytes can be inhibited by actinomycin D. It has also been confirmed that IF production by leucocytes from healthy donors is inhibited by puromycin (STRANDER, unpublished results) and by cycloheximide (HADHÁZY, STRANDER and CANTELL, unpublished results). We have extended the validity of these results to CML leucocytes (Table II).

Based on these findings it may be stated that also the Sendai virus-induced IF production by both normal and CML leucocytes needs *de novo* IF synthesis.

In accordance with our earlier experiments [12] we have concluded that serum is not required by the messenger RNA synthesis necessary for the

Sendai virus-induced IF production by normal human leucocytes *in vitro*, but translation does not take place in the absence of serum, probably due to ribosomal changes. Other authors [43], on the basis of experiments in a different system, arrived at a similar conclusion. The results published by VILCEK [44], VILCEK and NG [45], TAN *et al.* [46], HO *et al.* [47] and MYERS and FRIEDMAN [48] suggest that there exists an intracellular regulating (negative-controlling) mechanism of IF production acting post-transcriptionally, through a labile repressor of protein nature, supposedly at the level of translation. A similar mechanism might function in normal human leucocytes [15]. Indirect evidence of the existence in various cells of a factor regulating protein synthesis by the inhibition of polysomes has been reported by several authors [49-52].

Taking all these into consideration, we assume that (i) the presence of a serum factor(s) may, within certain limits, counteract the activity of a repressor acting at the level of translation on IF synthesis in normal human leucocytes; (ii) such a repressor is not formed, or is formed in a reduced amount, in human leukaemic leucocytes.

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SPECIFICITY OF ANTIGENIC FRACTIONS OF TUBERCULIN

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Summary. Purified protein derivative prepared from *Mycobacterium bovis* was separated into 6 fractions by electrophoresis at 1500 V/40 mA in pH 4.2 acetic acid-pyridine buffer. Further purification of one of the fractions on Sephadex G 25 column and by acid hydrolysis yielded antigen "PS" eliciting tuberculin reaction only in animals vaccinated with *M. bovis* BCG but not in those sensitized with *M. avium* and some fast-growing mycobacteria. The specificity of antigen "PS" was confirmed *in vitro*: the antigen induced blastic transformation only in lymphocytes of guinea pigs sensitized with *M. bovis* BCG.

In the past decades a number of data accumulated on the specificity and chemical composition of different tuberculin fractions [1-19]. The results indicated that the tuberculin fractions contain antigen complexes which, due to antigenic relationships between mycobacteria, may elicit an aspecific cutaneous reaction in animals sensitized with heterologous mycobacteria.

In the present study we have analyzed the antigenic components of bovine and avian PPD. We have attempted to prepare immunochemically pure components and to determine their specificity or cross-reactivity in animals.

Materials and methods

Fractionation of tuberculin was performed by means of medium voltage electrophoresis and column chromatography. For electrophoresis, Schleicher-Schuell No. 2024/a paper and sodium barbiturate or acetic acid-pyridine buffer were used; the samples were run at 1500 V/40 mA for 80 min.

The position of the fraction was determined with protein, lipide and polysaccharide staining [20]. Using repeated electrophoresis, each fraction was collected in sufficient amount to prepare tuberculin reagent (1 mg/ml) for animal experiments. One of the fractions was further purified on Sephadex G 25 column equilibrated with PBS or Tris buffer; the fraction obtained in this manner was hydrolysed with 0.1 N HCl.

Specificity of the fractions was examined *in vivo* and *in vitro*. For the infection of the animals different mycobacteria were cultured on Löwenstein-Jensen medium for 6 weeks. Guinea pigs were injected intraperitoneally with 0.5 ml volumes containing 0.05 mg *M. bovis* BCG or 0.5 mg *M. avium* GH (wet weight). Cattle were injected subcutaneously with BCG (5 mg wet bacteria in 2 ml volume); other strains were given orally (50 mg wet bacteria in 10 ml volume).

For the assay of blastic transformation the lymphocytes from heparinized blood specimens of sensitized guinea pigs were suspended in Parker's 199. To the suspensions (10^6 /ml) bovine PPD, avian PPD or antigen "PS" isolated from *M. bovis* AN₃ were added at 250 µg/ml. From the cell cultures samples were taken and stained at 48 and 72 hr intervals.

Results

Separation of PPD fractions. With electrophoresis at different pH values the tuberculin proteins were separated into fractions. Sodium barbiturate (pH 8.6) and acetic acid-pyridine (pH 5.6) buffers yielded 3 fractions from both kinds of PPD (Fig. 1). In acetic acid-pyridine buffer (pH 4.2) the bovine PPD was separated into 6 fractions of which 4 migrated towards the anode, 1

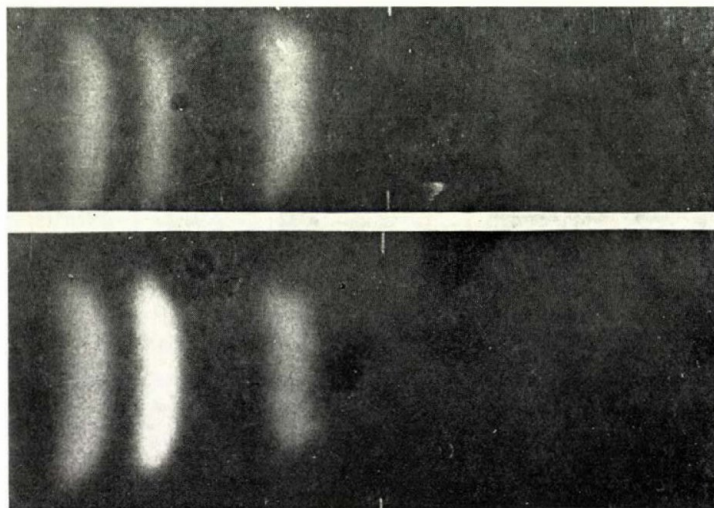


Fig. 1. Fractions of avian (upper row) and bovine (lower row) PPD; acetic acid-pyridine buffer (pH 5.6), 1500 V, 40 mA

towards the cathode and 1 was immobile. The avian PPD in the same buffer gave 5 fractions, 1 on the cathode side, 3 on the anode side and 1 remaining at the start point. Results obtained in pH 3.6 acetic acid-pyridine buffer are shown in Fig. 2.

The best separation, accordingly, was attained at pH 4.2. The activity of the 6 fractions eluted is presented in Table I. Fraction 3 of bovine PPD behaved differently in guinea pigs infected with *M. bovis* and with *M. avium*. This fraction contained proteins and polysaccharides; after removal of the latter by acid hydrolysis, the remaining fraction reacted only in guinea pigs sensitized with the homologous strain. This purified fraction was designated "PS" and used in subsequent experiments at 2 mg/ml concentration.

Experiments in cattle. The animals were infected with the mycobacterial strains shown in Table II. Three weeks after infection the tuberculin test was performed and repeated at 45-day intervals during 8 months.

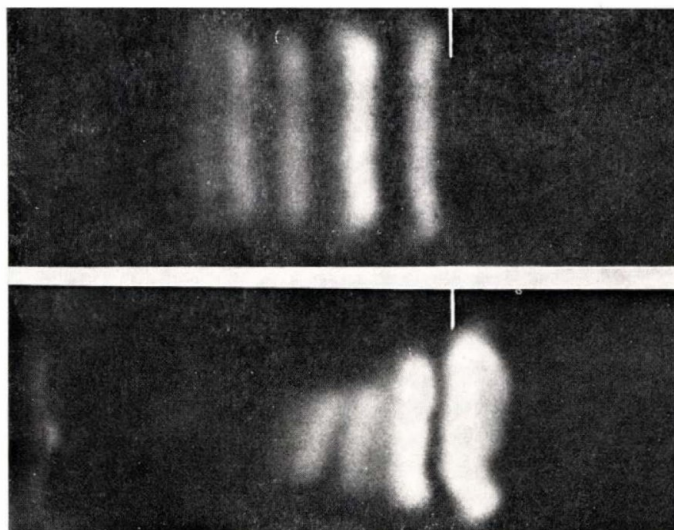


Fig. 2. Fractions of avian (upper row) and bovine (lower row) PPD; acetic acid-pyridine buffer (pH 3.6), 1500 V, 40 mA

Table I

Tuberculin reaction elicited in guinea pigs by electrophoretically separated fractions of bovine PPD

Strains used for infection	Reactivity of bovine PPD fractions*					
	1	2	3	4	5	6
<i>M. bovis</i> AN ₃	±	±	+	—	—	—
<i>M. avium</i> GH	—	±	±	±	—	—
Control	—	—	—	—	—	—

Fractions are designated in the order of position from the negative to the positive electrode

The results of tuberculin test carried out 3 months after infection is shown in Table II. All animals sensitized with BCG reacted with mammalian tuberculin and with the antigen "PS".

Mycobacteria of the avium group differed in sensitizing capacity. *M. avium* strains virulent to birds elicited a persistent reactivity to avian tuberculin from the 2nd month onward in 8 out of 10 cattle; the same animals reacted to mammalian tuberculin less frequently (3 out of 10) and only in the 3rd to 4th month. Cattle sensitized with Battley-type strains reacted to this antigen in about 40%; the allergic sensitivity persisted for 6 months similarly

Table II
Tuberculin reaction in cattle infected with various mycobacteria

Strains used for infection	No. of animals	Mammalian tuberculin			Avian tuberculin			"Ps" antigen		
		+	±	-	+	±	-	+	±	-
<i>M. bovis</i> BCG	10	10	—	—	3	1	6	10	—	—
<i>M. avium</i> serotype I	10	3	2	5	8	—	2	—	—	10
serotype II	10	4	—	6	8	—	2	—	—	10
serotype IV	5	1	—	4	3	—	2	—	1	4
Watson	5	1	—	4	2	—	3	—	—	5
P-39	5	2	—	2	1	2	2	—	—	5
<i>M. ulcerans</i>	5	1	—	4	1	2	2	—	—	5
<i>M. minetti</i>	5	1	—	4	2	2	1	—	—	5
<i>M. ranae</i>	5	1	2	2	2	2	1	—	—	5
Control	10	—	—	10	—	1	9	—	—	10

+ = positive, ± = doubtful, — = negative tuberculin reaction

as in animals infected with virulent strains. With mammalian tuberculin all these animals reacted uniformly in the 3rd to 4th month after infection.

From Table II it is evident that cattle infected with *M. avium* serotypes failed to react to antigen "PS". Sensitization with other mycobacterial species resulted in different degrees of reaction to mammalian and avian tuberculin but with an invariably negative result to antigen "PS".

Blastic transformation of lymphocytes of sensitized guinea pigs. The inductive effect of bovine and avian PPD was identical in animals injected with *M. bovis* (35%), but significantly differed in those sensitized with *M. avium* (16 and 42%, respectively). "PS" antigen induced transformation in lymphocytes of guinea pigs sensitized with *M. bovis* (40%) but not in lymphocytes originating from animals injected with *M. avium*.

Discussion

The active substance in tuberculin has long been known to be bound to proteins [21-23] and numerous attempts have been made at purifying this fraction. By electrophoresis, SEIBERT and GLENN [24] separated tuberculo-proteins into 3 fractions; these, however, showed no essential difference in immunodiagnostic tests. Similar results were described by other authors [16, 25]. By improved methods a more effective separation was carried out

[26-34] and in this manner a more precise determination of the antigenic properties of various tuberculin fractions has become possible [35-37]. Antigen fractions showing a strict species-specificity have, however, not been described.

Literary data suggest that various protein and polysaccharide fractions isolated with different preparative methods are, in fact, mixtures of a number of antigenic components [30, 33, 35, 37], and in intracutaneous test on animals infected with various mycobacteria they show merely quantitative differences.

The "PS" antigen isolated in the present study represents a fraction that, in contrast to other preparations, elicits tuberculin reaction in a specific manner only after *M. bovis* infection. The specificity of this antigen has been confirmed by the finding that lymphoblastic transformation was induced in the homologous system only.

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SEROLOGICAL DIFFERENTIAL DIAGNOSIS OF VIRAL HEPATITIS IN ADULTS

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Summary. Serum samples from different groups of adults were tested for HB_sAg and IH_xAg, using a complement-fixation microtest and the Indian-ink immune reaction, respectively. (i) In healthy men 18–24 years of age, living in camps in closed communities, HB_sAg was demonstrated in 1.5%, IH_xAg in 12.2%, and both antigens in 0.7%. The incidence of HB_sAg positivity seems to be age-dependent and influenced by environmental factors. (ii) For patients hospitalized with liver and/or biliary-tract diseases other than hepatitis, the respective percentages were 10, 13.5 and 4.5%. (iii) Of the cases clinically diagnosed as infectious hepatitis (IH, hepatitis A) or serum hepatitis (SH, hepatitis B), 14% were positive for both antigens whereas 10% were double-negative; 76% were positive for either HB_sAg or IH_xAg. In two-thirds of the single-positive cases the demonstrated antigen agreed with the clinical diagnosis, in one-third the unexpected antigen was present. (iv) SGPT and thymol turbidity values agreed better with the serological findings than with the clinical diagnosis. The number of days in hospital appeared to be related to both the serological findings and the clinical diagnosis. The clinical course was the most severe for those having both antigens in blood. (v) IH_xantibodies from early convalescence were sensitive, those from a later stage were resistant, to 2-mercapto-ethanol. (vi) No correlation was found between the presence of IH_xAg and that of the rheumatoid factor. (vii) The IH_x Indian-ink reaction is disturbed by the presence of labile serum proteins while the essentially similar reverse passive haemagglutination reaction was not affected by them. (viii) Testing for IH_xAg seems to be a procedure valuable in the differential diagnosis of IH and SH, though the results are less convincing in adult age than in childhood.

In a previous study [1] a good agreement was shown between the clinical diagnosis of serum hepatitis (SH, hepatitis B) and the presence in the blood of HB_sAg as demonstrated by the complement-fixation (CF) test. Subsequently, a microtest was developed for the demonstration of a presumably hepatitis A associated antigen (IH_xAg) the presence of which in children's sera appeared to be closely related to the clinical diagnosis of infectious hepatitis (IH, hepatitis A): the reaction was positive in 88% of children in the acute phase of IH. During convalescence the rate of positivity declined [2]. In convalescence sera an antibody (IH_xAb) inhibiting the IH_x Indian-ink microreaction was demonstrated. IH_xAg was partially purified by column chromatography and starch-block electrophoresis.

In the present work serial blood samples from adults suffering from viral hepatitis were tested for HB_sAg and IH_xAg. The incidence of the antigens was examined in relation to the clinical diagnosis and the clinical laboratory findings, with the aim of studying the specificity of the IH_x reaction.

Materials and methods

Patients. A total of 416 hospitalized patients was involved in the study. Of these, 177 patients (90 women and 87 men) suffered from liver and/or biliary-tract disease other than hepatitis. They ranged from 16 to 87 years in age with an average of 55.6 years. Most of them were icteric or subicteric at admission. Their distribution by clinical diagnosis is shown in Table 1.

In 123 cases (63 women and 60 men) the clinical diagnosis was IH. The women ranged in age from 14 to 63 years, the men from 16 to 80 years (31 on the average). The clinical diagnosis of IH was based on the principles developed by one of us [3]; according to these IH is characterized by an acute onset with fever and shivering. The spleen is palpable, the liver is enlarged. Gastrointestinal symptoms are frequently observed. History of contact with patient(s) suffering from IH can often be demonstrated.

The clinical diagnosis was SH in 116 cases including 56 women aged from 14 to 65 years and 60 men aged from 16 to 82 years. The mean age for these patients was 50 years. In these cases the onset was vague, [3] the symptoms progressed slowly, and articular complaints were common. The spleen was not, the liver was moderately enlarged. The majority had received transfusion(s) of blood or injections and/or had undergone some surgical intervention 45–180 days before the onset of illness. SGPT and serum bilirubin values were usually higher, the thymol turbidity results were lower, than in IH.

Healthy controls. These included 401 men 18 to 24 years of age, living in camps in closed communities.

Blood samples. Blood was taken for serological tests from the non-hepatitis cases on two occasions; from the patients suffering from viral hepatitis the first sample was taken in the first 5-day period after admission and, subsequently samples were taken at 10–14-day intervals. The double-blind principle was followed. From healthy controls one blood sample each was tested.

Serological methods. HB_sAg and anti-HB_sAg were titrated using a CF microtest [2, 4]. For the titration of IH_xAg and anti-IH_xAg the Indian-ink immune reaction was applied [2].

In addition, selected serum samples were tested by a reverse passive haemagglutination (RPHA) technique. For this purpose, packed sheep erythrocytes were suspended in 9 volumes of pH 7.3 veronal buffer, fixed in 4% glutaraldehyde solution for 25 hr and, after sedimentation and resuspension, in 2% formalin for another 24 hr. The erythrocytes were washed twelve times with isotonic saline, centrifuged and resuspended in 9 volumes of physiological saline. Then gamma globulin, of the same lot and in the same dilution as used in the Indian-ink reaction, was added.

The gamma globulin was allowed to adsorb to the erythrocytes by gentle shaking for 2 hr at room temperature. The suspension was kept at 4°C overnight. As stabilizer 4% normal bovine serum was added and the erythrocytes thus obtained were freeze-dried. The lyophilized substance was resuspended to make a 0.5% w/v suspension. Titration was performed in the U-shaped wells of Microtiter trays. One drop of the erythrocyte suspension was placed in each well and serial halving serum dilutions were prepared with the 25 μ l Microtiter loop in this suspension. Then a second drop of erythrocyte suspension was added and the tray was gently shaken in a horizontal mini-shaker at 37°C for 15 min. Subsequently, the erythrocytes were allowed to settle at room temperature for 3 hr.

Testing for nonspecific reaction. All serum samples tested for IH_xAg were in addition titrated with Indian-ink not pretreated with gamma globulin.

The eventual non-specific reaction between sheep erythrocytes and gamma globulin was controlled by parallel titration in "Hepanosticon absorbent" (sheep erythrocytes coated by sheep gamma globulin; Organon, Oss).

Clinical laboratory methods. These have been specified elsewhere [4]. The SGPT and thymol turbidity test were studied in detail.

Treatment of IH_xAb-positive sera with 2-mercapto-ethanol (2-ME). 2-Me stock solution (0.1 M) in veronal buffer (pH 7.3) [5] was added to 9 volumes of serum. In control tubes there was no 2-ME in the veronal buffer. The mixtures were allowed to stand at room temperature for 30 min, then the IH_x-inhibition test was performed. In 12 cases the first IH_xAg-negative samples, in three cases samples from a later stage of convalescence, were tested.

Rheumatoid factor (RF). Blood samples from 189 cases of acute hepatitis, 24 cases of chronic hepatitis proved by needle biopsy, and 49 healthy subjects were tested for RF. Serum samples were inactivated at 56°C for 30 min and the 1:20-diluted samples were tested for agglutination with Gamma latex reagent [6] (Institute for Serobacteriological Production and Research Human, Budapest).

Table I

Incidence of HB_s and IH_x antigenaemia in adult patients with liver diseases other than hepatitis

Clinical diagnosis	No. of cases	Positives for					
		HB _s Ag only		IH _x Ag only		both antigens	
		No.	per cent	No.	per cent	No.	per cent
Cholelithiasis, choledocholithiasis	58	5	9	8	14	3	5
Cholecystitis, cholangitis	66	9	14	6	9	2	3
Tumour including metastasis	5	1	.	1	.	1	.
Obstruction jaundice	13	1	.	1	.	1	.
Biliary dyskinesia	7	0	.	4	.	0	.
Steatosis	13	0	.	2	.	1	.
Cholestasis	3	1	.	0	.	0	.
Toxic hepatitis	6	0	.	2	.	0	.
Gilbert's disease	3	0	.	0	.	0	.
Hepatosplenomegaly	3	0	.	0	.	0	.
Total	177	17	10	24	13.6	8	4.5

Results

Incidence of HB_sAg and IH_xAg

Healthy men. Of the 401 men under study, in accordance with our earlier results [4], 6 (1.5%) proved to carry HB_sAg. The number of IH_xAg-carriers was 49 (12.2%) and, in addition, three men (0.7%) carried both. For unselected blood donors the rates of positivity had been lower [1].

Patients with liver and/or biliary-tract disease. Of the 177 non-hepatitis patients (Table I), 17 (10%) were positive for HB_sAg, 24 (14%) for IH_xAg, and 8 (4.5%) for both antigens. Two weeks after the first sampling, a second sample was examined from 104 cases. All the three percentage values showed some decrease (7.7%, 10.5% and 1.9%, respectively).

Patients with clinical IH. Distribution of the cases by the presence or absence of the two antigens in blood samples is shown in Table II. In the majority, the serological status remained unchanged throughout the observation period or until the reactions had become negative during convalescence; in 24 cases, the first finding was different from the later ones. Taking into account all single- and double-positive cases and all samples from each patient, IH_xAg was demonstrated in one or more samples from 96 cases (78%), whereas HB_sAg in 43 cases (34%). Both antigens were present in one or more samples in 10 cases, and 13 patients were invariably negative.

Patients with clinical SH. Of the 116 cases in this group (Table III) 70 (60%) had one or more serum samples positive for HB_sAg and 63 (54%) had at least one IH_xAg-positive sample.

Table II
Patients with clinical IH

Group No.	Antigens in serum				Cases		Inoculation in history		Possible extreme values for incubation, days	Average				
	1st sample		later samples							age, years	days in hospital	SGPT		thymol turbidity
	IH _x	HB _s	IH _x	HB _s	No.	per cent	No.	per cent				x	xx	
1	+	—	+	—	67	62	13	20	15-143	27	29	371	697	10
	+	+			9		2							
2	—	+	—	+	14	20	3*	22	40-150	34	33	555	1098	5
	+	+			9		2							
	+	—			1		0							
3	+	+	+	+	6	8	1	.	108-153	24	35	752	1203	10
	+	—			4									
4	—	—	—	—	13	10	0	.	—	45	23	239	320	5
Total					123	100	21	17	85	31	29	423	795	8.7

^x Means for all positive results; ^{xx} Means for first samples only

* One of these had continuously been exposed to hepatitis, incubation could not be calculated

Table III

Patients with clinical SH

Group No.	Antigens in serum				Cases		Inoculation in history		Possible extreme values for incubation days	Average				
	1st sample		later samples							age, years	days in hospital	SGPT		thymol turbidity
	IH _x	HB _s	IH _x	HB _s	No.	per cent	No.	per cent				x	xx	
1	+	—	+	—	23	25	16*	72	21–153	57	35	363	608	10
	+	+			3		2							
	—	+			3		3**							
2	—	+	—	+	30	32	23	76	45–240	45	36	556	892	6
	+	+			8		6							
	+	+			21		13***							
3	+	—	+	+	2	22	2	61	52–184	51	43	375	649	12
	—	+			3		1							
	—	—			23		17							
4	—	—	—	—	23	20	17	74	20–156	44	28	316	568	5
Total					116	100	83	71	20–240	50	35	419	723	8

x Means for all positive results; xx Means for first samples only

* Two of these were continuously exposed, incubation could not be calculated

** For one of these see *

*** For three of these see *

Age distribution, days in hospital, and laboratory findings

Patients with clinical IH. The IH_xAg -positive- HB_sAg -negative patients were on the average, less, the IH_xAg -negative- HB_sAg -positive ones were more, than 30 years of age. The serologically negative patients were mostly the oldest (45 years) and these spent the shortest time in hospital (23 days on the average). The average stay in hospital was substantially longer for the cases positive for HB_sAg or both antigens than for the IH_xAg positive cases.

Among the clinical laboratory reactions, the SGPT and the thymol turbidity tests showed some regularity. (a) The longer was the stay in hospital the higher were the SGPT values. (b) The average thymol turbidity level was 10 U for the IH_xAg -positive cases and 5 U for the negative ones. The presence of IH_xAg and the thymol turbidity level did not run parallel in the course of the disease. Occasionally, 12–18 U thymol turbidity values were associated with the absence, and a value of 2.2 U with the presence, of IH_xAg in serum.

Three patients each positive for IH_xAg remembered contact with IH patient(s) in the 40-day period preceding the onset of illness, and in 21 cases (17%) the illness was preceded by one or more transfusions of blood, injection therapy or surgical intervention. In 13 of these cases only IH_xAg was present in serial serum samples. The incubation periods calculated from the intervals between interventions and onset of illness were in general longer than expected in IH, and the extreme values for IH_xAg -positive and HB_sAg -positive cases covered each other, the lowest values having been shorter for the former.

Patients with clinical SH. The average age for each serological group was higher than 40 years. Those positive for IH_xAg or both antigens were older than 50 years on the average. The double-positives spent the longest, the double-negatives the shortest time in hospital. The HB_sAg -positive IH_xAg -negative cases had the highest average SGPT values, whereas the thymol turbidity values were the highest in the double-positive group. The SGPT and thymol turbidity values in the seronegative group, though relatively low, were still over the upper limits of normal values.

Inoculation was registered in 71% of the histories, and these cases were nearly equally distributed in the different serological groups. For patients having received serial inoculations for a long period of time, both the shortest and the lowest possible incubations have been calculated. Thus, the incubation periods for IH_xAg -positive cases ranged between 21 and 153 days. Part of the shortest possible incubation periods would have agreed with IH rather than with SH, the others were consistent with the clinical diagnosis.

Characteristic data for hepatitis patients grouped according to clinical diagnosis and antigenaemia. The SGPT and thymol turbidity values were more consistent as grouped on a serological basis (*i.e.* according to the presence of HB_sAg or IH_xAg) than as grouped by the clinical diagnosis (Fig. 1). Segregation of

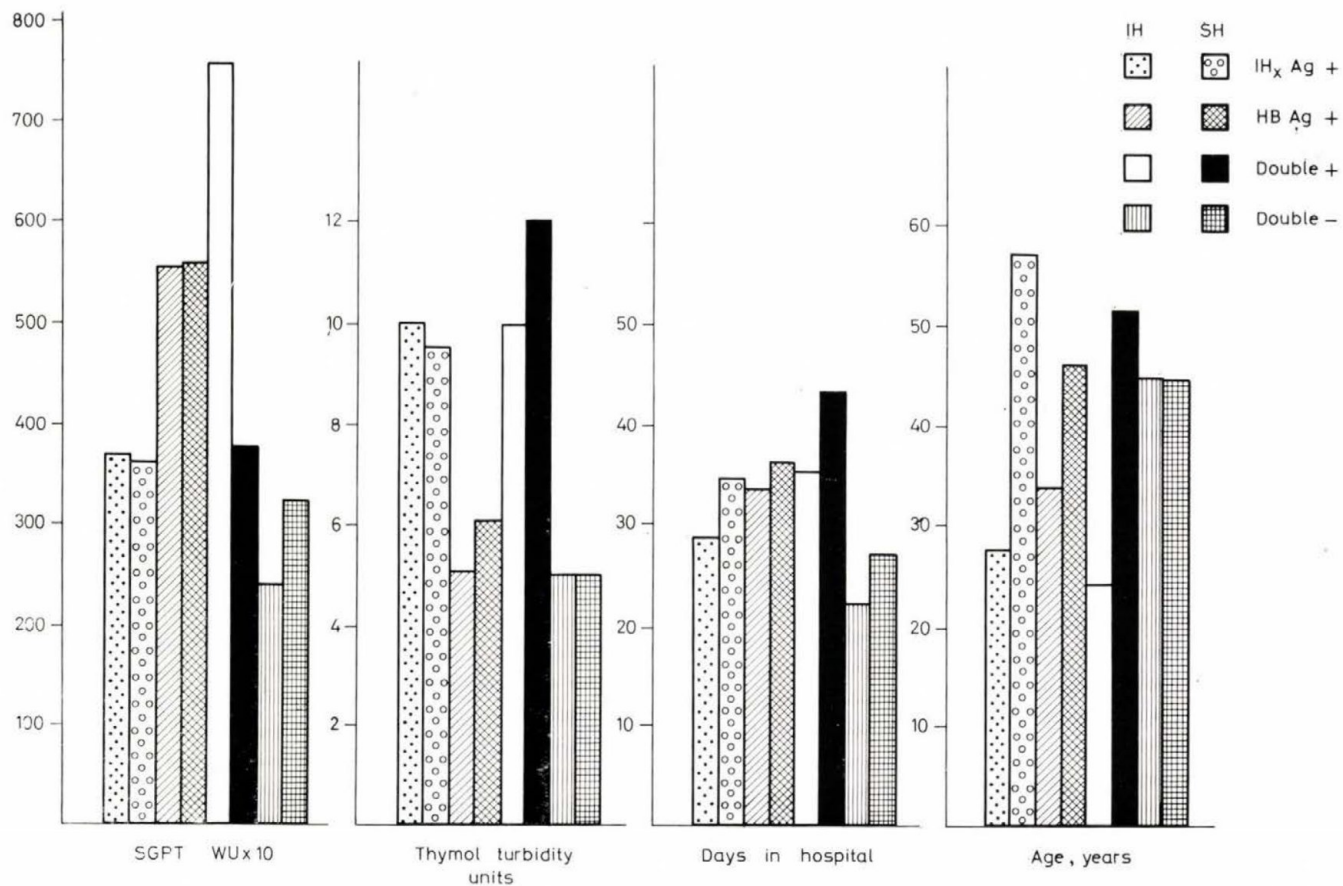


Fig. 1. Mean values for SGPT, thymol turbidity, days in hospital and age for hospitalized hepatitis patients grouped according to demonstrable antigenaemia

high (IH) and relatively low (SH) SGPT values by clinical diagnosis was pronounced in the group of double-positive cases only. The thymol turbidity values seemed to be unrelated to the clinical diagnosis even in this serological group. Accepting the average stay in hospital as an indicator of the severity of illness, the order of severity was double-positive-IH_xAg-negative HB_sAg-positive-IH_xAg-positive HB_sAg-negative-double-negative, in both clinical groups. Considering the great age difference between the two clinical groups in all serological groups except seronegatives, the patient's age might be a subjective factor in the clinical differentiation of IH and SH. The severe course of hepatitis in old age may have suggested the diagnosis of SH in certain cases. The age difference between IH and SH patients was the most pronounced in the serological group IH_xAg-positive HB_sAg-negative.

Non-specific Indian-ink aggregation. Three-hundred and thirty-three IH_xAg-positive blood samples from 146 subjects including 135 patients suffering from hepatitis and 11 from liver or biliary-tract disease were tested with uncoated Indian-ink for non-specific aggregation, which was expected to occur in the presence of labile serum proteins. Non-specific aggregation was observed in almost every case. The aggregation titre was lower than the IH_xAg titre (*i.e.* the IH_xAg-positivity was considered specific) in 47%, it was higher than the IH_xAg titre in 32%, and the two titres were equal in 21% of the samples.

The IH_xAg IIR was specific in all serum samples of 35 patients (24%). In 69 cases (47%) at least one sample had a higher IH_xAg titre than the non-specific aggregation titre, *i.e.*, 104 patients (71%) had higher specific than non-specific titre in one or more samples. In the remaining 29%, IH_xAg positivity could not be verified in this way.

It was therefore questionable whether these 29% of cases should be considered IH_xAg-positive. To elucidate this question, in the first serum sample available from 135 IH_xAg-positive patients the RPHA test was carried out. It was positive in each sample. The non-specific aggregation test failed to prove the specificity of the IH_xAg test in 39 cases (29%). However, in 27 cases, *i.e.*, in the majority of the doubtful ones, the RPHA test with sheep erythrocytes coated by sheep gamma globulin was negative or yielded a negligible titre. Since further samples were not available for RPHA testing, it seemed permissible to indicate in Fig. 1 and Tables II and III as positive, all the samples giving the IH_xAg Indian-ink reaction, irrespective of the result of the non-specific aggregation (Table IV).

2-ME sensitivity of IH_xAb. Pretreatment of 12 IH_xAb-positive early convalescent sera with 2-ME reduced the antibody titres to 1/4-1/10 of the original level. On the other hand, the three samples taken several months after convalescence showed hardly any titre fall (not more than 1:2). It is reasonable to suppose that in early convalescence IgM, later IgG IH_x antibodies predominate in the serum.

Table IV

Rheumatoid factor in patients with acute hepatitis and in healthy males

Group	Antigens in serum		No. of subjects	Positive for RF	
	IH _x	HB _s		No.	per cent
Healthy males	—	—	49	3	6
Adult patients with hepatitis	+	+	38	11	29
	+	·	31	2	
	Total IH _x +		69	13	19
	IH _x	HB _s			
	—	+	47	8	17
	—	—	73	21	29
	Total IH _x —		120	29	24
	Grand total		189	42	22

Table V

Comparison of Indian-ink reaction with RPHA in respect of disturbing non-specific reactions

		Sera giving positive reaction with SRBC coated by		Total
		human gamma globulin only	either human or sheep gamma globulin	
Sera giving positive reaction with	coated Indian-ink	94	2	96 (71%)
	both coated and uncoated Indian-ink	27	12	39 (29%)
Total		121 (90%)	14 (10%)	135 (100%)

SRBC = sheep red blood cells

RF in hepatitis sera (Table V). Of 49 double-negative healthy men 3 (6%), of the 189 hepatitis cases 42 (22%) were positive for RF. Although the latter percentage is considerably higher, RF seems to have no role in the IH_x reaction, for it occurred somewhat less frequently in IH_xAg-positive than in IH_xAg-negative cases.

Discussion

Among unselected blood donors, we found HB_sAg carriers in 1% [4] and IH_xAg carriers in 3%. Of the male donors aged 18–24 years, 1.5% carried HB_sAg. Until now we have not had any data for the age and sex distribution of IH_xAg carriers. In the present study both antigens were found more frequently in young men living in closed communities than previously demonstrated in unselected blood donors. The development of HB_sAg carriership is to some extent age and sex-dependent and influenced by environmental factors. According to the present results, IH_xAg carriership may be influenced by the same or similar factors.

In patients suffering from non-hepatic liver or biliary-tract disease, both HB_sAg and IH_xAg occurred more frequently than in blood donors (Table I). Perhaps, the liver lesion itself may produce conditions supporting antigen production and/or activating a latent infection. It cannot even be excluded that the production of these antigens may be due partly to a non-specific response to an infection or another noxa. During two weeks stay in hospital the frequency of both antigens declined.

Differentiation between SH and IH of adults encounters many difficulties, and one should be cautious in evaluating the results of the IH_xAg reaction as well. This may be disturbed by foreign antibodies present in the gamma-globulin preparation used as IH_xAb reagent for the demonstration of IH_xAg; furthermore, by labile lipoproteins non-specifically aggregating Indian-ink granules and by RF reacting with globulins. It was therefore justified to check the reliability of the IH_xAg from another aspect, *viz.*, studying the correlations with the clinical diagnosis, epidemiological data and results of the usual laboratory tests.

In a previous study [2], we examined for IH_xAg 177 children of whom 170 suffered from IH according to the clinical diagnosis. Of these, 88% were positive for IH_xAg. The remaining 7 children, diagnosed as SH, all proved to be HB_sAg-positive. Double-positivity occurred but transiently. These results supported the reliability of the IH_xAg reaction in the differential diagnosis of children's hepatitis. In the present adult material the relation of the serological results to the clinical diagnosis was less pronounced. Nevertheless, of the cases diagnosed clinically as IH, IH_xAg was demonstrated in 78%, and HB_sAg in only 34%, while the respective values for SH were 54% and 60%.

The disturbing effect of the non-specific aggregation is difficult to evaluate, especially if it consistently attains or surpasses the IH_xAg in titre. This occurred in 29% of the IH_xAg-positive cases in the present study.

The RPHA test, which was performed in a fraction of the IH_xAg-positive sera, was expected to be relatively insensitive to labile serum proteins. In accordance with this presumption, the rate of "nonspecific" positivities was

one-third of that obtained for the IH_x Indian-ink reaction. Since in the present study only IH_xAg -positive acute-phase serum samples were tested for RPHA, a comparison of the sensitivities of the two methods requires further study.

The 6.1% frequency of RF in healthy young men is in good accordance with the 5% positivity found in Hungary by BOZSÓKY [7] in miscellaneous clinical material. In hepatitis cases, RF was present at a considerably higher ratio [8, 9], but showed no positive correlation with IH_xAg positivity.

Of the antibodies inhibiting the IH_xAg reaction, those appearing in early convalescence seem to be IgM in nature, in contrast to the 2-ME-resistant, presumably IgG, antibodies demonstrable several months later.

About 20% of the hepatitis patients involved in the present study were positive for both HB_sAg and IH_xAg . These included the gravest cases. It would be a plausible explanation that these cases represent double infections with both hepatitis A and B viruses. Such a high frequency of simultaneous infections is, however, unlikely. Alternatively, in severe cases of viral hepatitis, components of liver origin might be incorporated into the surface antigens of both hepatitis A and B viruses. The incidence of double-positivity in the present adult material was not lower when the results of the RPHA test were considered instead of those of the IH_x Indian-ink reaction.

This explanation would be consistent with the literary data on HB_sAg positivity in IH cases [10, 11] and on particles of double antigenicity [12, 13]. It should be taken into consideration that low amounts of anti- HB_sAg in the gamma globulin used in the IH_x reaction may escape attention during the control of these preparations. This antibody may require large amounts of HB_sAg to give a positive reaction.

In spite of these possibilities, the majority of the serological results have been consistent with the clinical diagnosis, and in discrepant cases the results of the usual laboratory reactions agreed better with the serological results than with the clinical diagnosis.

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SEROLOGICAL INTERRELATIONSHIPS IN AVIAN MYCOPLASMA SEROTYPES

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Summary. A study of 18 standard avian mycoplasma serotypes and their antisera employing indirect haemagglutination test revealed distinct relationships among avian mycoplasma serotypes, so that they could be classified into 9 groups.

Although the avian mycoplasmas have been studied since 1933, their proper classification was lacking and interest has been focussed upon the distinction of pathogenic and non-pathogenic species without much emphasis on their antigenic interrelationships. Several investigators [1-7] have attempted to classify these species on the basis of their biological and serological properties. In 1964 YODER and HOFSTAD [8] classified them into 12 serotypes from A to L. In the following years DIERKS *et al.* [9] added to these 7 new serotypes, termed from M to S. In subsequent years, several investigators [10-12] felt that some of these serotypes might be mixtures of several species and performed a study to isolate them and to establish their characteristics. The indirect haemagglutination test (IHA) was used as it was suggested [13] to be the most specific and sensitive serological test for the identification of human mycoplasmas. By its means we have reinvestigated the serologic interrelationship of the 18 serotypes A to R.

Materials and methods

Mycoplasma strains. Reference strains of avian mycoplasma representing 18 serotypes A to R were obtained from Dr. M. L. FREY, Iowa State University, Iowa, USA. EDWARD's modified medium [14] was used to grow these strains to prepare the immunizing antigens. Each strain was cloned three times from a single colony before the studies were undertaken.

Immunological procedures. Antigens of each of the above 18 serotypes were prepared according to DIERKS *et al.* [9].

Hyperimmune serum against each of the above strains was prepared in albino rabbits according to TAYLOR-ROBINSON *et al.* [15]. Preimmunization blood samples were collected from each rabbit and tested for the presence of natural agglutinins against each strain before using them. Only non-reactors were used.

The IHA test described by LIND [16] with a slight modification [17] was used. The modification was that the sheep erythrocytes were not formalinized; the other procedures were unchanged.

Table I

Serological interrelationship of reference serotypes of avian mycoplasma using indirect haemagglutination test

Reference antigens with strain number	Reference antisera*																	
	A	B	M	C	O	D	P	E	G	F	H	I	J	K	N	Q	R	L
A 801	5120																	
B 862		20480	2560															
M R49		1280	1280															
C 859				1280	640													
O TC-5				160	5120													
D 8						2560	160											
P TC-3						320	5120											
E C-26								40960	1280									
G 867								640	1280									
F SA										40960								
H 8M92											1280							
I 695												2560	80	160	80	80	40	
J 693												640	2560	80	40	40	640	
K 1805												160	80	1280	80	40	40	
N PHN-D 13												80	80	160	1280	80	40	
Q L3-10												80	20	40	80	1280	40	
R D2497												320	0	80	80	80	5120	
L 694																		2560

* Reciprocal of antibody titres with homologous and heterologous antisera

Results

The IHA titres of the serotypes with homologous and heterologous antisera are presented in Table I. Serotypes A, F, H and L did not show cross-agglutination with any of the antisera used except with homologous antisera. Cross-agglutination between serotypes B and M, C and O, D and P and with a complex group of the six serotypes I, J, K, N, Q and R was observed. The IHA titres of serotype B with antisera B and M were 20,480 and 2560, respectively, whereas of serotype M the titre with homologous and heterologous antisera was 1280 and 1280, respectively. Similar observations were made with the serotypes E and G, C and O, D and P and in the group of six serotypes though their titre with heterologous antisera was lower than with homologous antisera.

Discussion

The results showed that serotypes A, F, H and L are, in agreement with other findings [8-12, 18, 19], distinct antigenically since they did not show heterologous agglutination with antisera other than homologous ones. With IHA test, serotypes E and G, and B and M were identical and did not give cross-agglutination with other antisera, confirming the findings [8, 11] that suggested that serotypes E and G, and B and M were one and same, termed *Mycoplasma iners* and *M. gallinarum*, respectively. Antigen M elicited a poor antibody response though antibodies developed to the same extent as with antigen B. Therefore these two serotypes seem to be identical. Serotypes C and D showed some antigenic overlapping with serotypes O and P, respectively, since these serotypes gave cross-reactions with heterologous antisera. There were considerable cross-reactions between the 6 serotypes of the complex group, in contrast to the findings of DIERKS *et al.* [9] who considered them antigenically distinct on the basis of plate agglutination and growth-inhibition tests. Our findings using IHA test confirmed the findings of FREY [20] who employed complement fixation test. It would not be unjustified to consider the complex group of the 6 antigenic serotypes as one group based upon their biochemical identity. These findings were confirmed by BARBER and FABRICANT [12] using metabolic inhibition test. On the basis of the above, we suggest a regrouping of the avian mycoplasma serotypes as follows. Serotypes A, F, H and L should be regarded as distinct types of *M. gallisepticum*; the following 8 serotypes are joined to form 4 distinct groups: B + M (*M. gallinarum*), E + G (*M. iners*) C + O and D + P; the biochemically identical 6 serotypes are classified into one separate group.

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ANTIBACTERIAL EFFECT OF SOME PHENOTHIAZINE COMPOUNDS AND R-FACTOR ELIMINATION BY CHLOROPROMAZINE

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Summary. Chlorpromazine, levopromazine and promethazine exerted a bacteriostatic effect on Gram-positive bacteria at 20–60 $\mu\text{g/ml}$, on Gram-negative bacteria at 130–180 $\mu\text{g/ml}$ concentration. Of the three compounds, chlorpromazine had the most marked bactericidal effect on cultures of *Bacillus anthracis* growing in minimal medium. In addition, chlorpromazine had a significant bactericidal effect on the resting cells of *Escherichia coli* suspended in saline. *Pseudomonas aeruginosa* was resistant to phenothiazines. Experiments have failed to derive resistant mutants from the highly sensitive *B. anthracis*. An effective R-factor elimination was observed at chlorpromazine concentrations of 50 $\mu\text{g/ml}$, practically not affecting the growth of multiple resistant *E. coli*.

Chlorpromazine, a well-known tranquillizer exerts a wide variety of effects on biochemical processes. Some of its actions, studied so far on mammalian cells, are as follows. The drug produces an allosteric block of glutamate dehydrogenase [1–3], inhibits succinoxidase, succinodehydrogenase and cytochromoxidase [4], decreases the plasma insulin level [5], acts as an anti-tumour agent [6] and hinders calcium accumulation and respiration in the mitochondria of cerebral cells [7].

Literary data for the antibacterial effect of phenothiazines are, in many respect, contradictory. Some authors deny the direct antibacterial effect and claim to have shown that chlorpromazine merely potentiates the activity of antibiotics [8]. Others regard the compound as a bacteriostatic [9] or bactericidal [10, 11] agent. Data for the bactericidal concentration of chlorpromazine vary considerably even with the same bacterial species [10, 12, 13]. The discrepant results may be attributed to methodical or technical differences. The purpose of the present paper was to study the antibacterial effect of chlorpromazine, promethazine and levomepromazine under standard conditions.

Materials and methods

Drugs. Chlorpromazine (Hibernal®), promethazine (Pipolphen®), levomepromazine (Tisercin®) E. Gy. T., Budapest; ethidium bromide, Serva, Heidelberg.

Bacterial strains. *Bacillus anthracis* VR, *Escherichia coli* B, *Streptococcus pyogenes*, *Streptococcus viridans*, *Staphylococcus aureus*, *Corynebacterium hofmanni*, *Diplococcus pneumo-*

niae, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*. Except the first two organisms, the strains were isolated in our laboratory from clinical specimens. Elimination of R-factor was examined in *E. coli* K12 J5 RC 709 *F*⁻ *prol*⁻ *meth*⁻ *Sm*^r *T*^r *Su*^r *Cl*^r kindly supplied by Dr. N. DATTA, London.

Media. *B. anthracis* was grown on lactalbumin hydrolysate medium (LA) completed with glucose, aneurin, salts and certain amino acids [14]. In part of the experiments yeast-extract-peptone (YP) medium [15] and in part broth and blood agar were used. Elimination of R-factor was examined in MTE medium [16].

Antibacterial spectrum was examined with the agar diffusion method. Twenty ml of nutrient or blood agar were poured into Petri dishes 90 mm in diameter and seeded with broth cultures of 0.1 OD. Five wells, each 8 mm in diameter, were cut and filled with 50 μ l solution containing 12, 25, 125, 250 and 1250 μ g of the phenothiazine compounds. After incubation at 37°C overnight the zones of inhibition were measured.

The degree of bacteriostatic effect was tested by the use of the tube dilution method in broth containing 0.4% yeast extract. Serial dilutions of the drugs (0–200 μ g/ml) were prepared in 5 ml volumes and seeded with 0.1 ml portions of the 1 : 10 dilution of 0.1 OD exponential phase cultures; the c.f.u./ml values of 0.1 OD cultures were $4.1\text{--}5.2 \times 10^7$ for *E. coli* and $2\text{--}8.2 \times 10^7$ for *S. aureus*. Minimal inhibitory concentrations were read after 18 hr incubation at 37°C.

Bactericidal effect was examined by inoculating 0.1 ml aliquots of overnight pre-cultures into 10 ml LA, YP or MTE media. The cultures were aerated in water bath at 37°C until they had reached an OD of 0.35, then different amounts of phenothiazine were added. During further incubation the cultures were sampled at 15 min intervals; they were examined with Gram and Gutstein's wall stain and their optical density was determined at 620 nm in the Bausch and Lomb Spectronic 20 photometer.

Elimination of R-factor was examined as described earlier [17] with the following modifications. An overnight pre-culture of *E. coli* K12 was diluted 10^{-4} and distributed at 0.05 ml aliquots (approx. 10^3 bacteria) into tubes with 5 ml MTE broth. The culture was supplemented with chlorpromazine (50 μ g/ml) and incubated without shaking at 37°C for 72 hr. Then dilutions of the culture were seeded at 0.1 ml amounts onto YP plates. After incubation overnight, the isolated colonies were replicated with velveteen disc onto YP plates containing different antibiotics (streptomycin, tetracycline and chloramphenicol each 50 μ g/ml; sulphadimidine, 400 μ g/ml). After incubation at 37°C overnight, the number of colonies was compared with the number of colonies growing on the master plate. Control cultures were treated in a similar manner with 100 μ g/ml of ethidium bromide [18].

Colonies not appearing at the corresponding site of the antibiotic plates were transferred from the antibiotic-free plate into saline to give OD = 0.01, seeded again onto antibiotic and control plates and incubated at 37°C for 24–72 hr. R⁻ bacteria were checked for auxotrophy.

Results

Earlier it was found that chlorpromazine at 31.8 μ g/ml inhibited the germination of *B. anthracis* spores in liquid medium. In the present studies the growth of Gram-positive bacteria, as tested with the agar diffusion method, was inhibited by chlorpromazine and levomepromazine at 12–25 μ g, by promethazine at 125 μ g, amounts. Gram-negative strains were less sensitive and *P. aeruginosa* was not inhibited even by 1250 μ g of the drugs (Table I). The minimum inhibitory concentrations of the three drugs for different bacteria in fluid medium are shown in Table II. The three compounds showed no difference in bacteriostatic concentration; Gram-positive organisms proved to be more sensitive than Gram-negative ones and *P. aeruginosa* grew at concentrations as high as 1000 μ g/ml.

In subsequent experiments the bactericidal effect of the three phenothiazines was examined. The drugs were added at 31.8 μ g/ml final concentra-

Table I

The antibacterial effect of chlorpromazine, levomepromazine and promethazine on different organisms examined with agar diffusion method

Bacterial strains	Chlorpromazine μg					Promethazine μg			Levomepromazine, μg			
	1250	1250	1250	250	250	125	125	25	25	102	12	12
<i>B. anthracis</i> VR	35	32	30	18	12	23	18	12	26	20	18	
<i>Corynebacterium hofmanni</i>	26	22	20	16	—	22	18	11	not tested			
<i>Diplococcus pneumoniae</i> *	30	24	22	15	12	25	14	10	25	18	17	10
<i>Staphylococcus aureus</i>	26	22	19	11	11	20	17	14	25	19	15	10
<i>Streptococcus pyogenes</i> *	29	23	12	—	—	25	18	16	24	20	17	11
<i>Streptococcus viridans</i> *	30	25	12	—	—	27	22	19	21	17	15	—
<i>Escherichia Coli</i> B	18	14	12	10	—	24	13	11	18	12	10	—
<i>Klebsiella pneumoniae</i>	13	11	10	—	—	14	11	—	13	10	9	—
<i>Proteus vulgaris</i>	15	13	—	—	—	14	—	—	12	10	—	—
<i>Pseudomonas aeruginosa</i>	—	—	—	—	—	—	—	—	—	—	—	—

Figures indicate the diameter of inhibition zones in mm

* Tested on blood agar

Table II

Bacteriostatic effect of chlorpromazine, promethazine and levomepromazine on different organisms examined with the tube dilution method

Bacterial strains	Minimal inhibitory concentration, $\mu\text{g}/\text{ml}$		
	chlorpromazine	promethazine	levomepromazine
<i>Bacillus anthracis</i> VR	30	40	40
<i>Corynebacterium hofmanni</i>	40	40	40
<i>Diplococcus pneumoniae</i>	20	30	30
<i>Staphylococcus aureus</i>	40	60	50
<i>Streptococcus pyogenes</i>	30	40	40
<i>Streptococcus viridans</i>	40	50	50
<i>Escherichia coli</i> B	130	150	140
<i>Klebsiella pneumoniae</i>	140	160	180
<i>Proteus vulgaris</i>	160	150	160
<i>Pseudomonas aeruginosa</i>	>1250	>1250	>1250

tion to exponential phase *B. anthracis* culture. After the addition of chlorpromazine the optical density gradually decreased and finally almost all cells were lysed. Promethazine and levomepromazine at the same concentration had practically no effect on the growth of *B. anthracis* (Fig. 1). In cultures incubated with chlorpromazine the damaged cells became Gram-negative; the injurious effect was demonstrable also with wall staining (Plates I and II). As seen in

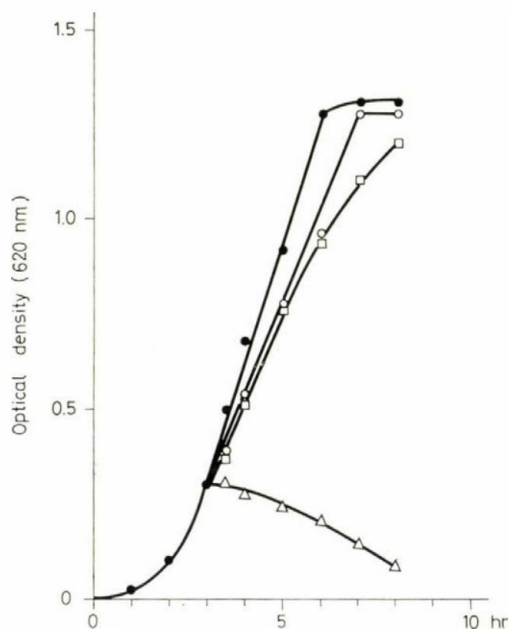


Fig. 1. Effect on growth of *B. anthracis* in lactalbumin medium (control, ●—●) of chlorpromazine (△—△), levomepromazine (□—□) and promethazine (○—○) at 31.8 $\mu\text{g/ml}$ concentration

Fig. 2, the antibacterial effect of chlorpromazine in liquid culture was associated also with the number of microorganisms present.

Chlorpromazine had damaged and even destroyed resting *E. coli* B and *S. aureus* cells in saline. The direct injurious effect was more marked for *S. aureus* at 37°C the original 3×10^7 count decreased by 2.5 exponents 60 min after the addition of the drug (80 $\mu\text{g/ml}$), whereas for *E. coli* the decrease amounted to one exponent only.

Next 250 $\mu\text{g/ml}$ of chlorpromazine was added to exponential phase cultures (OD = 0.2) of *E. coli* B growing in MTE broth and the culture was incubated under shaking at 37°C. Optical density (Fig. 3) and living counts (Fig. 4) of the culture were determined at intervals. Although *E. coli* cells

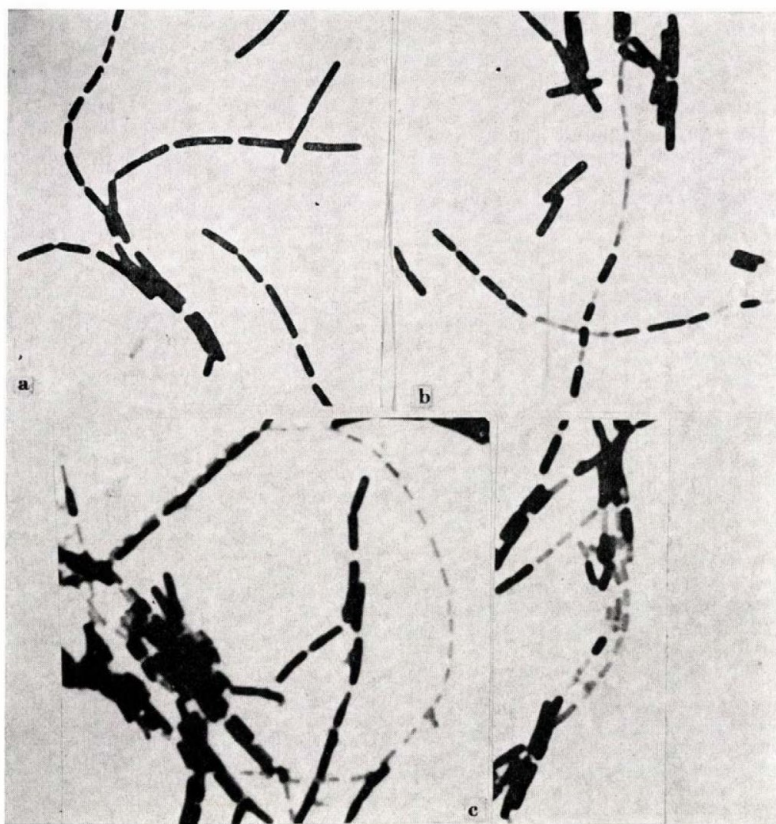


Plate I. Gram staining of *B. anthracis* grown in lactalbumin medium (a) 0 min, (b) 60 min, (c) 120 min after the addition of chlorpromazine (31.8 $\mu\text{g/ml}$). $\times 2700$

failed to show complete lysis, at the 120th min of incubation no surviving bacteria were detected.

The effect of chlorpromazine at different concentrations is shown in Fig. 5. The drug was added to a culture of $\text{OD} = 0.35$; at 31.8 $\mu\text{g/ml}$ concentration the optical density increased somewhat for 15–30 min, then gradually decreased. At 63.6 $\mu\text{g/ml}$ the initial increase was absent.

Our efforts have failed to make *B. anthracis* VR strain resistant to chlorpromazine.

Elimination of R-factor by chlorpromazine was examined in multiple antibiotic resistant *E. coli* K12. Eighty-one out of 547 colonies grew and were subculturable only on antibiotic-free plates. The control culture, treated with ethidium bromide, lost its antibiotic resistance in a somewhat lower ratio



Plate II. Gutstein's cell wall staining of *B. anthracis* grown in lactalbumin medium (a) 0 min (b) 60 min, (c) 120 min after the addition of chlorpromazine (31.8 $\mu\text{g/ml}$) . $\times 2700$

(25 out of 240 colonies). The R^- bacteria derived either after chlorpromazine or after ethidium bromide treatment, retained their characteristic properties. Control experiments showed no spontaneous loss of R-factor.

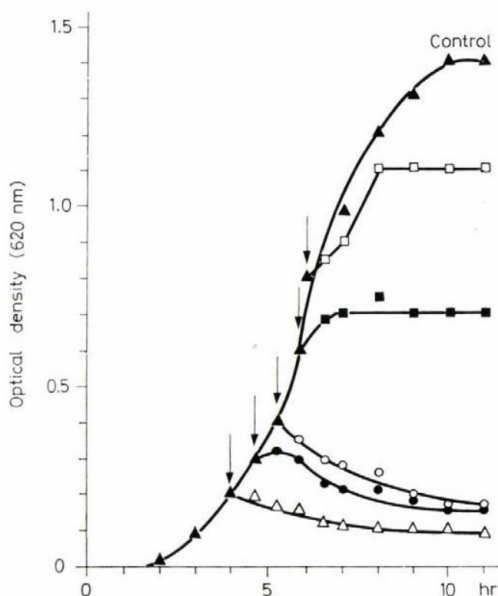


Fig. 2. Effect of chlorpromazine at 95.4 $\mu\text{g/ml}$ concentration on *B. anthracis* cultures of various optical density in lactalbumin medium. The arrows indicate the addition of chlorpromazine to the cultures

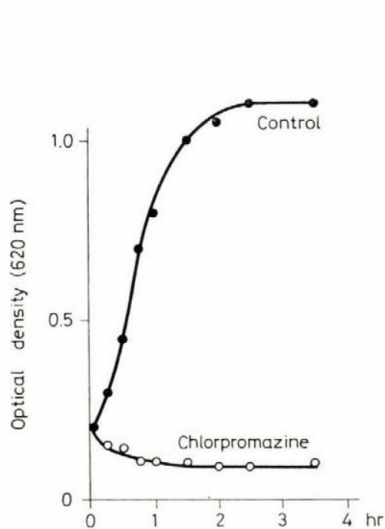


Fig. 3. Effect of chlorpromazine (250 $\mu\text{g/ml}$) on *E. coli* B grown in MTE broth

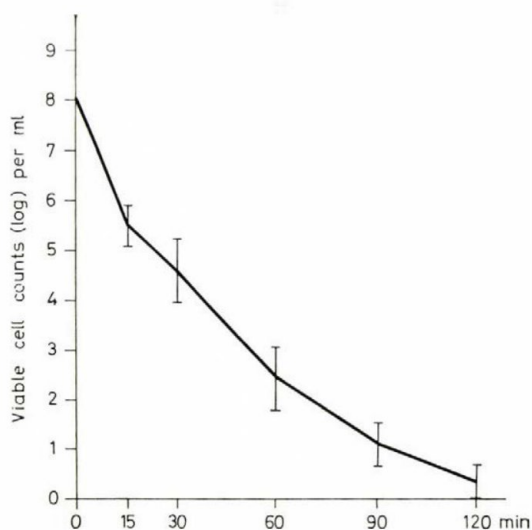


Fig. 4. Bactericidal effect of chlorpromazine (250 $\mu\text{g/ml}$) on multiplying cells of *E. coli* B

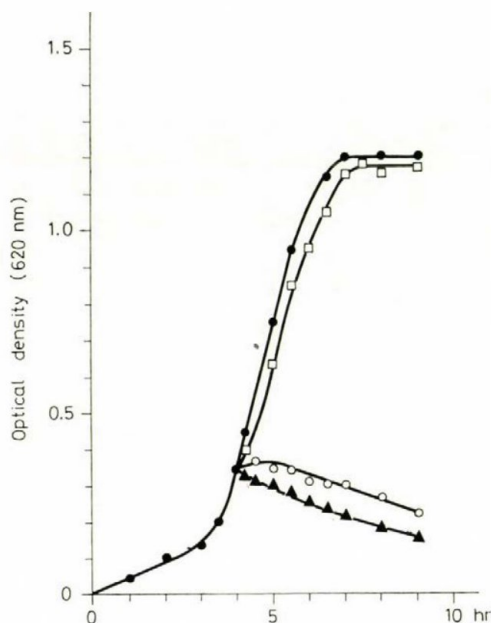


Fig. 5. Effect of chlorpromazine at different concentrations on the growth of *B. anthracis* in lactalbumin medium. ●—● control; □—□ 15.9 µg; ○—○ 31.8 µg; ▲—▲ 63.6 µg; chlorpromazine per ml

Discussion

In mammalian cells chlorpromazine damages the cell membrane [6, 19 20] and inhibits the function of many membrane-bound enzymes [11]. Phenothiazines may alter the permeability of biomembranes and inhibit oxidative phosphorylation [19]. It has been assumed that chlorpromazine acts by inhibiting SH enzymes [22]. The compound inhibits the germination of *Bacillus megaterium* spores; this may be associated with the inhibition of hexose-monophosphate dehydrogenase [23], but for the mechanism of antibacterial effect we still have no sufficient data.

In the antibacterial or bactericidal action, in addition to the direct membrane effect, the structure of the bacterial cell wall may play an important role. Chlorpromazine was bactericidal to a staphylococcal and a streptococcal strain at 50 µg/ml, whereas to *Clostridium perfringens* and *E. coli* only at 5000 µg/ml [13]. The present experiments showed that Gram-negative bacteria possessing cell wall rich in lipids were less sensitive to phenothiazines including chlorpromazine than Gram-positive organisms. These findings are in agreement with data indicating that chlorpromazine acts mainly on Gram-positive bacteria [10]. Since bacterial cultures are partly or totally lysed in the presence of chlorpromazine, impairment of the cell wall seems probable; cell wall

and Gram staining supported this assumption. While BOURDON [10] failed to show a difference in chlorpromazine sensitivity of *E. coli* and *P. aeruginosa*, in our experiments the former proved to be more sensitive than the latter. A certain degree of resistance to chlorpromazine can be induced artificially. In serial passages the resistance of *Streptococcus faecalis* was increased from 10 to 40 $\mu\text{g/ml}$ [10]. In our experiments *B. anthracis* strain VR was trained to grow in the presence of 30–40 μg chlorpromazine per ml. The resistance obtained in this manner could not be increased further and in chlorpromazine-free medium the strain regained its original sensitivity (unpublished data).

From the fact that different phenothiazines exert an antibacterial activity, it may be assumed that compounds effective in much lower concentrations can be produced. As to the use of phenothiazines as antibacterial drugs *in vivo*, there are data that by intramuscular administration 0.5–5.0 $\mu\text{g/ml}$ serum levels can be reached, which are lower than the bacteriostatic concentration [9]; in contrast, others recommend high plasma levels (150–300 ng/ml) for treating certain psychiatric diseases [24]. Nearly 40 years ago it was shown that oral administration of phenothiazines to rats, rabbits and to patients results in an excretion bactericidal concentrations in the urine [25]. On the basis of these findings phenothiazine was successfully used for treating urogenital infections; prolonged treatment, however, impaired the haemopoiesis [11].

In view of the side effects of chlorpromazine, its antibacterial activity is of theoretical interest.

Our observation on the elimination of R-factor from 15% of multiple resistant *E. coli* cells is also of theoretical interest. Under the same conditions ethidium bromide yielded a 10.5% elimination; other data for the latter substance with two different *E. coli* strains ranged between 32 and 71% [18].

Chlorpromazine probably damages several biochemical pathways not only in mammalian but also in bacterial cells. This assumption is supported by our failure in training *B. anthracis* to chlorpromazine resistance.

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NON-SPECIFIC ANTIMICROBIAL CELLULAR RESISTANCE IN SENSITIZED GUINEA PIGS

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Summary. Specific delayed-type hypersensitivity was induced in guinea pigs with bovine albumin + complete Freund adjuvant, bovine gamma globulin + complete Freund adjuvant and BCG vaccine. The animals were subsequently tested for nonspecific antimicrobial resistance. Sensitized and control groups were challenged intraperitoneally with *Listeria monocytogenes* 2 hr after reinjection with the sensitizing antigen. The listeria content of the spleens was determined 1 or 5 days after the infection. The number of organisms recovered from the spleen one day after infection was significantly less in guinea pigs sensitized with bovine gamma globulin and BCG than in the control group; after 5 days no such difference was recorded. There was no difference between the bovine albumin sensitized and the control group 1 day after infection, while on the 5th postinfection day listeria counts were higher in the sensitized than in the control animals.

In an earlier study [1] we investigated the relationship between delayed type hypersensitivity to a simple protein and nonspecific antimicrobial resistance in guinea pigs. The animals were sensitized with 100 μ g of bovine albumin (BA) in Freund's complete adjuvant (CFA). Specific skin sensitivity developed 4 days after the antigen injection; a significant inhibition of migration of the peritoneal cells became evident after 14-17 days. Antibodies to BA demonstrated with passive haemagglutination appeared in rising titres from the 7th day onward. Cellular resistance of sensitized animals was examined by challenging them intraperitoneally with *Listeria monocytogenes*. One day after challenge the sensitized and control animals' spleen contained the same number of organism, whereas 5 days later the germ count was higher for the sensitized animals than for the controls. Accordingly, sensitization with BA elicited no increased anti-infectious resistance; as judged from the 5-day results, the sensitized animals proved less resistant than the controls. These findings disagreed with the data of some authors who claimed to have shown an increased resistance to listeria infection in guinea pigs sensitized with bovine gamma globulin (BGG) [2]. It was supposed that the difference might be attributed to the use of two different kinds of antigen or breed of guinea pigs. In subsequent experiments, accordingly, the development of delayed hypersensitivity, the course of antibody production and resistance to listeria infection after CFA+BGG injection were examined. As a well-known model for studying the relationship between delayed type hypersensitivity and nonspecific resist-

ance, BCG-vaccinated tuberculin positive guinea pigs were also tested for resistance to listeria. Testing of BA-sensitized animals was repeated in this study; the data presented here derive mainly from the previous experiments and partly from new ones.

Materials and methods

Antigens. Bovine albumin fraction V (Browning Chemical Corporation), freeze-dried bovine gamma globulin (Phylaxia, Budapest) and freeze-dried BCG vaccine containing 10^6 – 3×10^6 viable units per ml after rehydration (National Institute of Public Health, Budapest).

Adjuvant. Freund's complete adjuvant (Difco).

Challenging organism. *L. monocytogenes* NCTC 7973.

Animals. Random-bred guinea pigs of both sexes weighing 300–350 g were used.

Sensitization. Physiological saline solutions of BA and BGG (1000 $\mu\text{g/ml}$) were mixed with equal volumes of CFA. From the mixture 0.05 ml was injected into each of the four foot pads of each guinea pig. The BCG vaccine was rehydrated with distilled water and 5×10^5 viable units were injected intramuscularly; 3–4 weeks after BCG vaccination a positive skin reaction at least 10 mm in diameter was elicited by intracutaneous injection of 10 IU PPD.

The course of specific delayed hypersensitivity and specific antibody production were examined in guinea pigs sensitized with CFA + BGG. At intervals after sensitization skin test, migration inhibition test and titration of serum antibodies to BGG were performed. These methods have been described in detail previously [1].

Skin test. BGG (100 μg) was injected intracutaneously and the result was read after 24 hr.

Migration inhibition test. Peritoneal cells obtained after injection of liquid paraffin were washed and collected in capillaries. After centrifugation the part of the capillary containing the cell column was fixed to the bottom of a chamber devised from a glass slide, a plastic ring and a coverslip. Peritoneal cells of each animal were examined in 5 control chambers (containing Parker's 199 with 20% normal guinea pig serum) and in 5 antigen chambers (containing the same medium + 500 μg BGG per ml). After incubation at 37°C for 24 hr the migration areas were projected onto a sheet of paper, cut out and weighed. The result was considered positive if the average weight of migration areas in the antigen chambers was not more than 70% of the mean of the controls (MI 70%).

Titration of antibodies to BGG. Sheep erythrocytes were tanninized, sensitized with BGG (1000 $\mu\text{g/ml}$) and preserved with formalin [1]. Sensitivity of the suspension was checked with anti-bovine gamma globulin rabbit serum (Human, Budapest). Passive haemagglutination titres were determined by TAKÁTSY's micromethod.

Measurement of the resistance to listeria infection. Guinea pigs sensitized 3–4 weeks earlier and untreated guinea pigs of the same weight were injected intraperitoneally with the corresponding antigen (1000 μg BA, 1000 μg BGG or 5×10^5 viable unit of BCG). Two hours later the animals were challenged intraperitoneally with 10^6 – 2×10^6 *L. monocytogenes* cultured for 48 hr on serum agar and washed in saline before injection. Part of the animals was sacrificed 1 day, part of them 5 days after challenge and the number of listeriae in the spleen was determined by culturing on blood-agar plates.

Results

1. *Course of specific delayed hypersensitivity and antibody production in guinea pigs sensitized with BGG.* Table I shows that specific skin sensitivity had developed 5 days after sensitization and was still demonstrable on the 28th day. In Fig. 1 it can be seen that sensitivity of peritoneal cells to BGG appeared 6–13 days, and became marked 13–17 days, after injection; on the 28th day the migration inhibition was still significant (MI 70%). Passive haemagglutinating antibodies appeared 5 days after BGG injection; the titres rose to significant levels by the 10th day. On the 30th day, very high titres were recorded (Table II).

Table I

Development of specific skin sensitivity in guinea pigs injected with BGG

Interval between BGG injection and testing (days)	No. of animals	Distribution of animals according to maximum diameter of skin reaction, mm			
		<5	5-10	11-20	>20
3	6	6	—	—	—
5	5	—	—	6	—
12	7	—	7	—	—
28	6	—	2	3	1

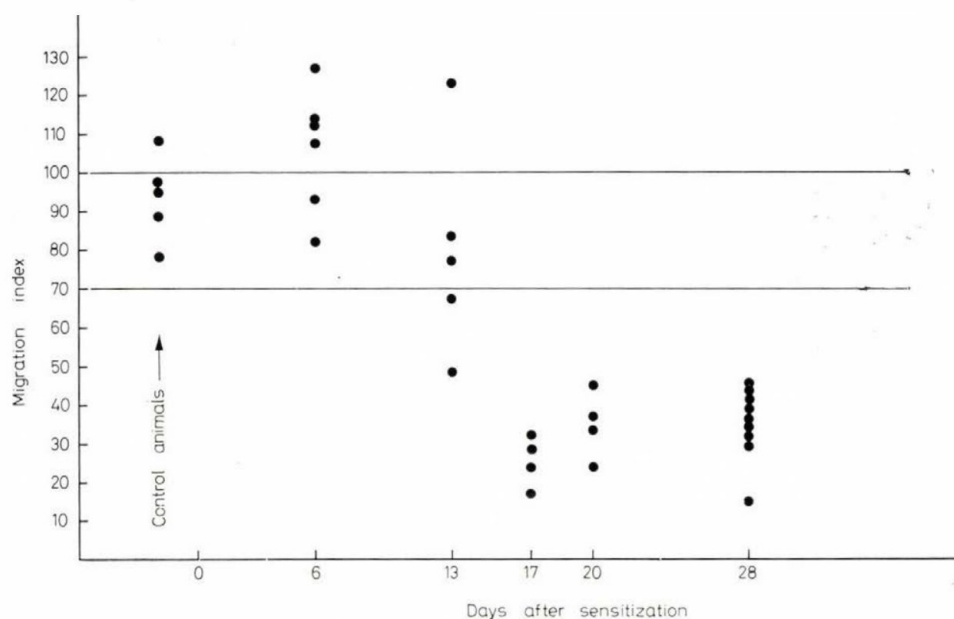


Fig. 1. Development of antigen-induced migration inhibition in guinea pigs sensitized with BGG

Table II

Production of antibodies in guinea pigs sensitized with BGG

Interval between BGG injection and testing, days	No. of animals	Distribution of sera according to reciprocal log ₂ passive haemagglutination titres													
		<2	2	3	4	5	6	7	8	9	10	11	12	13	14
0	6	6	—	—	—	—	—	—	—	—	—	—	—	—	—
5	6	3	2	1	—	—	—	—	—	—	—	—	—	—	—
10	7	—	—	—	—	—	—	2	2	3	—	—	—	—	—
30	6	—	—	—	—	—	—	—	—	—	—	—	1	4	1

2. *Resistance to L. monocytogenes of guinea pigs sensitized with BA, BGG and BCG.* At least 3 repeated experiments were performed on sensitized and control groups each consisting of 6–10 animals. The data are summarized in Tables III, IV and V.

(a) The spleens of guinea pigs sensitized with BA and of control animals were compared for listeria counts 1 day and 5 days after challenge in 5 and 6 different experiments, respectively. As shown in Table III, there was no significant difference between the two groups at the 1 day testing ($\chi^2_3 = 3.073$, $p > 0.05$); after 5 days significantly higher counts were more frequent in the sensitized than in the control group. The difference was significant ($\chi^2_2 = 18.646$, $p < 0.001$).

(b) Spleens of guinea pigs sensitized with BGG together with those of the controls were examined 1 and 5 days after challenge, in 3 different experiments each. On the day following listeria infection lower bacterial counts occurred more frequently in the sensitized animals than in the controls ($\chi^2_2 = 11.577$, $p < 0.01$). The 5-day values showed no significant difference ($\chi^2_3 = 5.919$, $p > 0.05$), although higher counts were more frequent in the sensitized group (Table IV).

Table III

Number of listeriae recovered from the spleen of BA-sensitized and control guinea pigs 1 and 5 days after challenge

Group	Interval between challenge and testing, days	No. of animals	Distribution of animals according to bacterial counts			
			$< 10^1$	$10^1 - 10^2$	$10^2 - 10^3$	$> 10^3$
Sensitized	1	38	1	4	18	15
Non-sensitized		38	1	1	15	21
Sensitized	5	42	0	11	17	14
Non-sensitized		42	9	15	16	2

Table IV

Number of listeriae recovered from the spleen of BGG-sensitized and control guinea pigs 1 and 5 days after challenge

Group	Interval between challenge and testing, days	No. of animals	Distribution of animals according to bacterial counts			
			$< 10^1$	$10^1 - 10^2$	$10^2 - 10^3$	$> 10^3$
Sensitized	1	28	0	10	11	7
Non-sensitized		28	0	1	10	17
Sensitized	5	26	1	10	13	2
Non-sensitized		23	4	13	5	1

Table V

Number of listeriae recovered from the spleen of BCG-sensitized and control guinea pigs 1 and 5 days after challenge

Group	Interval between challenge and testing, days	No. of animals	Distribution of animals according to bacterial counts			
			<10 ¹	10 ¹ –10 ²	10 ² –10 ³	>10 ³
Sensitized	1	29	5	11	13	0
Non-sensitized		29	0	4	12	13
Sensitized	5	27	5	10	12	0
Non-sensitized		29	2	14	9	4

Table VI

Number of listeriae recovered from the spleen of guinea pigs sensitized with different antigens

Group	Interval between challenge and testing	No. of animals	Distribution of animals according to bacterial counts			
			<10 ¹	10 ¹ –10 ²	10 ² –10 ³	>10 ³
Control	1	95	1	6	37	51
	5	94	15	42	30	7
Sensitized with BA	1	38	1	4	18	15
	5	42	0	11	17	14
Sensitized with BCG	1	28	0	10	11	7
	5	26	1	10	13	2
Sensitized with BCG	1	29	5	11	13	0
	5	27	5	10	12	0

(c) Three different experiments were made on BCG-sensitized and control animals (Table V). One day after challenge the bacterial counts in the sensitized group shifted towards lower values as compared to the controls; the difference was significant ($\chi^2_3 = 21.307$, $p < 0.001$). The 5-day values were, however, similar in both groups ($\chi^2_3 = 6.317$, $p > 0.05$).

Table VI was prepared from the data of Tables III, IV and V to demonstrate the difference between bacterial counts on the 1st and on the 5th day postinfection, separately in the sensitized and in the untreated animals.

In the above series of experiments altogether 95 and 94 control animals were examined 1 and 5 days after challenge, respectively. *Listeria* counts were high after 1 day (above 10²) in 92.6% of the animals. On the 5th day such high values were shown only in 39.3%. The difference was highly significant ($\chi^2_3 = 73.357$, $p < 0.001$).

Guinea pigs sensitized with BA showed similar bacterial counts 1 day (38 animals) and 5 days (42 animals) after challenge; ($\chi^2_3 = 4.082$, $p > 0.05$).

Practically the same values were recorded for BGG-sensitized guinea pigs sacrificed 1 day and 5 days after challenge (28 and 26 animals, respectively): $\chi^2_3 = 3.875$, $p > 0.05$.

Guinea pigs injected with BCG behaved similarly as those sensitized with the two other antigens; spleen counts showed no difference 1 day (29 animals) and 5 days (27 animals) after challenge ($\chi^2_2 = 0.015$, $p > 0.05$).

Discussion

From the present results it may be assumed that the nature of the sensitizing protein plays a decisive role in the relationship between delayed hypersensitivity and nonspecific antimicrobial resistance. Data of the previous [1] and the present paper indicate that both BA and BGG-sensitized guinea pigs develop specific delayed-type hypersensitivity as demonstrated by skin tests and migration inhibition tests. In addition, both proteins give rise to high titre specific antibodies. There was no essential difference in the development of these immunological parameters.

In contrast, resistance to *L. monocytogenes* infection differs in BA-sensitized and in BGG-sensitized animals. In the spleen of the former, the number of listeriae 1 day after challenge is practically the same as in the controls, while in BGG-treated guinea pigs the listeria counts are significantly lower as compared to untreated animals.

Specific delayed hypersensitivity and acquired antimicrobial resistance are, according to the widely accepted theory of MACKANESS [3], unseparable from each other. Certain findings, however, disagree with this assumption. RAFFEL [4] then YOUNG and YOUNG [5] described that tuberculin sensitivity can be elicited in animals with certain products of *Mycobacterium tuberculosis* without increasing their resistance to infection. Recently OSEBOLD *et al.* [6] have shown that in mice vaccinated with *L. monocytogenes* specific antibacterial resistance was independent of delayed hypersensitivity to the components of the agent. The present findings indicate that delayed hypersensitivity to soluble proteins and nonspecific acquired antimicrobial resistance are also different immunological phenomena.

From Tables IV and V it is evident that an increased resistance to listeriae of BGG- and BCG-sensitized animals is demonstrable only on the day following challenge. Five days after infection the spleen counts were similar in the test and in the control animals. Accordingly, antimicrobial resistance, at least under the conditions of the present experiments, persists for a short time. This short-term resistance is followed by a depressed immune state: as seen in Table VI, in the spleen of untreated animals the number of listeriae had decreased considerably by the 5th day, whereas in sensitized guinea pigs the 1-day and 5-day values were practically the same. It is remarkable that the

1-day and 5-day values were similar after sensitization with BA which induced no resistance. Thus the increased short-term and the subsequent depressed antibacterial immune state develop on the basis of different mechanisms.

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EFFECT OF SUGARS, HYDROGEN ION CONCENTRATION AND AMMONIUM NITRATE ON THE FORMATION OF CITRIC ACID BY *ASPERGILLUS NIGER*

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Summary. *Aspergillus niger* Mulder strain when grown on a synthetic medium containing urea as the sole source of nitrogen at pH 5.2, formed a mixture of citric and gluconic acids. On growing the organism at pH 2.0 the gluconic acid content was reduced but citric acid yield remained low. Addition of NH_4NO_3 to the medium lowered the gluconic acid yields to undetectable levels with a simultaneous increase in the citric acid content. Of the sugars used for the production of citric acid, sucrose in an unautoclaved medium was found to be the best carbon source. Sucrose medium if autoclaved at pH 2.0, or a mixture of glucose and fructose instead of sucrose gave lower yields of citric acid. Under optimum conditions only citric acid was produced and the yield was 66–68 g per litre after a growth period of about 10 days.

Citric acid is widely used as an acidulant in food and beverage industries. Almost all commercial citric acid is manufactured by fermentation process using molds (or recently yeasts) on synthetic or complex media. It is not common that large amounts of citric acid should accumulate in the growth media. The formation of oxalic and gluconic acids along with citric acid is another major problem [1, 2–6]. The present study was undertaken to determine the effect of pH, nitrogen sources and sugars both on the yield and nature of the organic acids formed.

Materials and methods

Organism. *Aspergillus niger* Mulder strain, Wageningen was purchased from Central Bureau Voor Schimmelcultures, Baarn, Netherlands and used throughout this study. The mold was maintained and subcultured on a sterile agar substrate containing NaNO_3 , 3.0 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KCl, 0.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g; sucrose, 30.0 g; Difco agar, 15.0 g; and distilled water to make one litre.

Production of citric acid. The spore suspension of the organism was inoculated into a seed medium [7] which had the following composition (per litre of distilled water): sucrose, 150 g; urea, 1.0 g; KH_2PO_4 , 0.08 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g; KCl, 0.15 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.02 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g. Fifty ml of this medium were dispensed into 500 ml capacity flasks (with 2 baffles) and were shaken at 200 rpm at 28°C for 4–5 days. The seed culture developed into uniform round pellets of mycelium and was transferred in 20 ml portions into 500 ml flasks containing 80 ml of the production medium of KAROW and WAKSMAN [7] with the following composition (per litre of distilled water): sucrose, 150 g; urea, 0.5 g; KH_2PO_4 , 0.05 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g; KCl, 0.15 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.02 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g. The flasks were maintained under conditions similar to those for the seed culture. Production of citric acid during and after the fermentation was followed by the colorimetric procedure of MARIER and BOULET [8].

Production of organic acids by varying pH and N-sources. The basal seed and production media had a pH around 5.2. The effect of acid conditions on citric acid production was studied by lowering the pH to 2.0 with 1 N HCl in both the media. The N-source in the two

media was urea. In a separate experiment the effect of NH_4NO_3 at concentrations of 0.34 to 4.0 g per litre at pH 2.0 on the production of citric acid was also studied.

Production of organic acids using various sugars. The basal medium (containing NH_4NO_3 at pH 2.0) contained 15% sucrose as the carbon source. The effect of various sugars on citric acid production was studied by replacing sucrose with glucose, fructose, lactose or maltose in an unautoclaved medium. The yield obtained was compared with sucrose in an autoclaved medium. Mixtures of glucose, sucrose and fructose were also tried as the carbon sources in an unautoclaved medium.

Paper chromatography of sugars and organic acids. Paper chromatography of sugars was performed on Whatman's filter paper No. 1 by the descending method [9] using a solvent containing butanol: acetone : water (2 : 7 : 1). The spots were located with alkaline AgNO_3 .

Citric, gluconic and oxalic acids were separated by ascending chromatography on Whatman's filter paper No. 1 using a formic acid, water and butanol (2 : 5 : 12) solvent system. The mixture was incubated at room temperature for 2 weeks before use to avoid tailing and double spots. After the incubation, two phases were formed; the water phase was used for saturation of the chromatography chamber for at least 24 hr, and the organic phase was used for separation. The chromatogram after development was dried in an oven at 120°C for 60 min. The spots were detected using ethanolic 0.04% bromocresol green containing sufficient 0.1 N NaOH until a blue colour had appeared. Yellow spots of the organic acids appeared against a blue background when the chromatogram was dipped in the above reagent.

Results

Effect of pH and NH_4NO_3 on the yield of organic acids. By using the production substrate of KAROW and WAKSMAN [7] which contained urea as the nitrogen source pH (5.2), a mixture of gluconic and citric acids (42 and 46 g per litre, respectively) was formed (Table I). Adjusting the pH to 2.0 with HCl

Table I

Effect of hydrogen ion concentration on the production of citric acid and gluconic acid

pH of the growth medium	Yields of acids (g/l)	
	Citric acid	Gluconic acid
5.2	46*	42*
2.0	41.5	29.0

* These figures are average of triplicate samples

reduced the amount of gluconic acid to 29 g per litre but the citric acid level remained low (41.5 g per litre).

On the addition of NH_4NO_3 at pH 2.0 the nitrogen source increased from zero to 2.0 g per litre; this increased the citric acid yield but reduced the gluconic acid to undetectable levels (Table II). Increasing the NH_4NO_3 concentration beyond 2.0 g per litre did not increase the yield. The colour of the mycelium changed from yellow to orange in the presence of NH_4NO_3 during fermentation.

Effect of sugars on the yield of citric acid. When the organism was grown on medium containing NH_4NO_3 at pH 2.0 and sucrose as carbon source, and if no autoclaving was done, the samples obtained at different intervals for sugar analysis showed (Plate I) that sucrose was completely inverted into glu-

Table II*Effect of ammonium nitrate at pH 2.0 on the production of citric and gluconic acids*

Concentration of NH_4NO_3 (g/litre)	Yields of the acids (g/litre)	
	Citric acid	Gluconic acid
0.0	48*	33*
0.34	59	8
1.00	62	10
1.60	64	3
2.00	66	0
2.40	62	0
2.80	65	0
3.20	65	0
4.00	64	0

* These figures are averages of triplicate samples

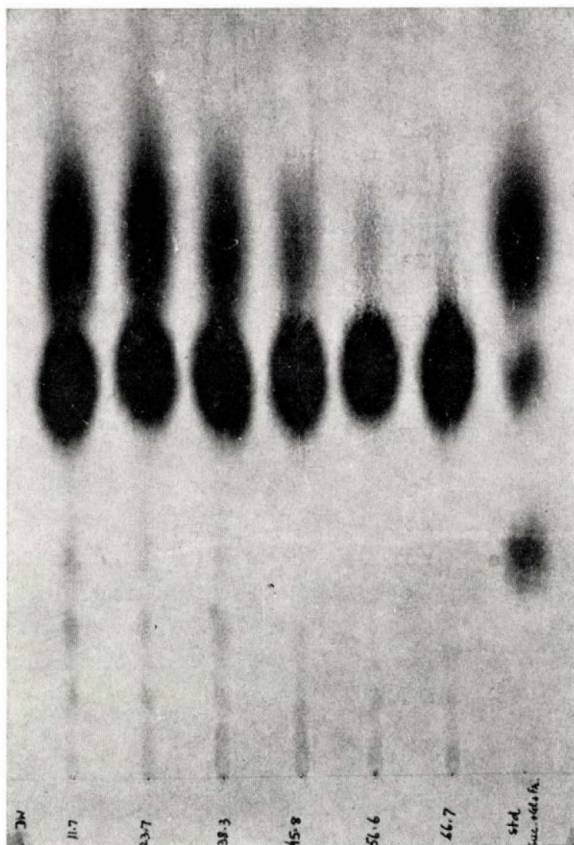


Plate I. Paper chromatogram showing sucrose, fructose and glucose in broth at different intervals of the fermentation when the organism was grown under optimum conditions for citric acid production. At right, a mixture of the three standards (from top to bottom) fructose, glucose and sucrose. Six samples were obtained during the fermentation period. The figures under the sites of application represent citric acid (g per litre) in the spotted sample

cose and fructose approximately after 48 hr of incubation. At this stage the yield of citric acid was about 11.0 g per litre. It was observed that fructose disappeared from the medium faster as the citric acid levels increased.

In a separate experiment, the organism was grown on fructose, sucrose, glucose, lactose, mixtures of glucose-sucrose and of glucose-fructose, all unautoclaved, and compared with sucrose in autoclaved medium. Maximum citric acid yield was 61.0 g per litre; this occurred in unautoclaved sucrose medium (Table III). Fructose alone or in combination with glucose; and

Table III
Effect of different sugars on the yield of citric acid

Sugar(s) used	Concentration (g/litre)	Yield of citric acid (g/litre)
Sucrose	150	61.0*
Glucose	150	17.0
Sucrose + glucose	10 + 140	18.0
Sucrose + glucose	100 + 50	33.5
Fructose	150	10.0
Glucose + fructose	75 + 75	25.5
Sucrose (autoclaved medium)	150	23.0
Lactose	150	<1.0
Maltose	150	2.0

* These figures are an average of three samples

autoclaved sucrose medium produced 10.0, 25.5 and 23.0 g per litre of citric acid, respectively. The growth of the organism in lactose medium was poor and no citric acid was produced. Mixtures of sucrose and glucose resulted in lower yields of citric acid and the decrease was related to the content of glucose in the medium.

It is clear from above that the role of fructose is rather ambiguous. Although sucrose was completely hydrolyzed in 2 days and fructose disappeared faster when the accumulation of citric acid had started, sucrose still proved to be a better source of carbon than the mixture of glucose and fructose or fructose alone. Similarly high yields of citric acid were not obtained from autoclaved sucrose medium when sucrose was inverted into glucose and fructose by acid-heat treatment.

Discussion

The low yields of citric acid as well as the additional formation of oxalic and gluconic acids are problems in the production of citric acid. *A. niger* Mulder strain when grown on a basal medium devoid of NH_4NO_3 at pH 5.2 resulted in the formation of both citric and gluconic acids. No oxalic acid was ever detect-

ed. An adjustment of the pH to 2.0 depressed the gluconic acid level but there was no corresponding increase in the yield of citrate. Gluconic acid was probably produced by a simple reaction of glucose oxidase with glucose (formed from inverted sucrose) and the activity of the enzyme was reduced at pH 2.0. This medium contained urea as the source of nitrogen and on the addition of 2 g per litre of NH_4NO_3 the gluconic acid was reduced to negligible levels with a simultaneous increase in the yield of citric acid to 66.0 g per litre. It could not be established whether the gluconic acid pathway had been blocked or it used up rapidly in the fermentation. SHIMI and MOUSTAFA [5] too reported an improvement of citric acid yield on the addition of NH_4NO_3 . On the other hand, KNO_3 was observed to increase the yield of oxalic acid [2].

Sucrose in an unautoclaved medium proved to be the best source of sugar for the production of citric acid. After the first 48 hr of fermentation, when the corresponding yield of citric acid was about 11 g per litre, the sucrose was completely inverted and with further growth fructose was consumed faster than glucose as the citric acid accumulated in the medium. Fructose alone and fructose and glucose in a 50 : 50 mixture gave, however, lower yields and the same was the case with an autoclaved sucrose medium; thus resulted in the inversion of sucrose at pH 2.0. The coupling of the inversion of the sucrose before its utilization by the organism with the high yields of citric acid seems to suggest that the loci for the invertase and citrate-synthesizing enzymes are closely located; so that when the former is produced the latter are also produced. Another possibility is that glucose might act as catabolic repressor if we start on a glucose medium or on an autoclaved sucrose medium or glucose-fructose medium.

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DEMONSTRATION OF ADENOVIRUS ASSOCIATED ENDONUCLEASE

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Summary. In HEp-2 and amnion cell cultures infected with type 1 adenovirus the DNase activity of cell extracts was measured on ^{32}P -labelled *Escherichia coli* DNA substrate. Enzyme activity was demonstrated by the acid soluble nucleotides released from the ^{32}P -DNA and by the decreased sedimentation rate of labelled DNA. High DNase activity was measured in both adenovirus infected and in untreated HEp-2 cell extracts. Nuclease activity of the amnion host cells being much lower than that of HEp-2 cells, virus-associated endonuclease activity was successfully demonstrated in them. Purified type 1 adenovirions decreased the sedimentation rate of ^{32}P -labelled *E. coli* DNA. The phenomenon is explained by the virion-associated endonuclease activity. Trypsin inactivated and anti HEp-2 IgG failed to inhibit the virion nuclease. An association between endonuclease and trypsin sensitive penton is assumed.

BURLINGHAM and DOERFLER [1] in 1972 demonstrated in type 2 and 12 adenovirus-infected cells the presence of endonuclease which decomposed the adenovirus DNA, by splitting the DNA molecule of about 23×10^6 dalton weight into 5 fragments of nearly identical size. The enzyme was detected not only in the extract of infected cells, but also in intact adenovirions purified by ultracentrifugation [1]. Of the virion capsomeres it was found to be bound to the penton [2].

Literary data suggested that adenovirus type 1 contained similar endonuclease activity. The present study was aimed at demonstrating the enzyme in infected cells and purified virions, using a ^{32}P -labelled *E. coli* DNA substrate produced by a simple technique.

Materials and methods

Virus strain. A laboratory strain of type 1 adenovirus was used.

Cell cultures. 1. HEp-2 monolayer tissue cultures were propagated in 2 litre bottles, using Parker's 199 solution with 15% calf serum and antibiotics. 2. Primary amnion cultures were prepared from fresh placenta in 2 litre bottles using Hanks's solution containing 20% human serum and lactalbumin.

Virus titration on HEp-2 monolayer cultures was evaluated by the method of REED and MUENCH [3].

Preparation of cell extracts. HEp-2 monolayer cultures were infected with 100 TCID₅₀ of virus and incubated at 37°C for 48 hr. Uninfected cell cultures were used for control. Cells were collected and washed three times in isotonic NaCl containing 0.01 M phosphate buffer (pH 7.2). Suspensions containing 5×10^7 cells/ml buffer were prepared and disintegrated by freezing-thawing. The material was centrifuged for 1 hr at 100,000 g at 1°C in an SW 56 rotor, using a

Beckman L 265 B ultracentrifuge. Subsequently, the sediment and the supernatant were separated, the sediment was resuspended in 0.01 M isotonic NaCl-phosphate buffer (pH 7.2). The protein concentration of the cell extract was adjusted to the same value.

Purification of adenovirions was performed with the technique of GREEN and PIŠA [4] with the following modification. HEP-2 monolayers were infected with 100 TCID₅₀ of virus and harvested after 48 hr. After washing three times in 0.01 M isotonic $\frac{1}{2}$ NaCl-phosphate buffer pH 7.2, the cells were subjected to freezing and thawing and centrifuged at 2000 g for 10 min. The supernatant was layered on a 1.41 g/cm³ CsCl cushion and centrifuged for 1 hr at 46,000 g at +1°C in a SW 41 rotor. The virion fraction was separated and purified with three subsequent equilibrium centrifugations 1.34 g/cm³ CsCl; 0.01 M Tris HCl pH 8.0; 80,000 g, 1°C, SW 41 rotor, 11 hr. The purified virion preparations were dialyzed against 0.01 M phosphate buffer (pH 7.2), at 4°C for 48 hr. One ml of pure virus contained 10¹¹ TCID₅₀.

Isolation of ³²P-labelled E. coli DNA was performed with the technique of ANAI *et al.* [5] in our modification. *E. coli* B strain was cultured till the end of the logarithmic phase on Bacto peptone medium [6] in the presence of 35 μ Ci/ml of ³²P at 37°C. Cells were centrifuged and washed with distilled water (0°C) and twice with a solution containing 0.5% NaCl and 0.5% KCl. Bacteria were suspended in a 0.15 M NaCl + 0.1 M EDTA solution (pH 8.0; ml/g wet weight of bacterium), and after adding 10 mg/g cell of lysozyme, the suspension was incubated at 37°C for 30 min.

Three hundred μ g of pronase per g cell were added to the material and incubation was continued for 15 min. Subsequently, 10 ml/g cell of 1% Na dodecylsulphate, 0.1 M Tris and 0.1 M NaCl were mixed to it and the material was kept at 60°C for 15 min. Fresh distilled phenol saturated with 0.1 M Tris HCl solution (pH 9.0) was added in identical volume to the disintegrated cells. After 20-min extraction, the material was centrifuged at 3500 g for 15 min and the aqueous phase was collected. The precipitate situated at the border of the two phases was extracted with an identical volume of Tris-phenol as described above and the two aqueous phases were pooled. A double volume of ethanol (0°C) was layered on the material and the nucleic acid precipitate was suspended in 9 ml/g cell of a 0.015 M NaCl + 0.0015 M Na citrate solution. After adding 1 ml/g cell of 1.5 M NaCl + 0.15 M Na citrate, the suspension was incubated at 37°C for 30 min with 2 mg/g cell of RNase (DNase-free, treated at 80°C for 10 min). After adding 1 ml/g cell of a mixture of 3 M Na acetate and 0.001 M EDTA, isopropanol (0°C) was layered on the material in 3/4 volume. The nucleic acid precipitate was suspended in NaCl-Na citrate mixture as described above, and the precipitation by isopropanol was repeated. The precipitate was suspended again in NaCl-Na citrate solution. The acid soluble ³²P was usually 0.5% or less.

Demonstration of nuclease activity. (a) The reaction mixture (0.3 ml) contained 10 μ g of ³²P-labelled *E. coli* DNA (10⁵ cpm) and the assumed enzyme in 0.01 M Tris-HCl, 0.002 MgCl₂, 0.1 M NaCl buffer (0°C, pH 7.2).

(b) *Demonstration of acid soluble nucleotides.* After incubation at 37°C for 0, 5, 15, 30 and 60 min, 0.2 ml of 0.5 mg/ml of bovine serum albumin and 0.5 ml of 5% perchloric acid was added to the above reaction mixture at 0°C. After 10 min standing the material was centrifuged at 2000 g at 1°C for 10 min; 0.5 ml of the supernatant was placed in a scintillation cuvette and the radioactivity was measured.

(c) *Sedimentation profile of the DNA* was determined with the method of BURLINGHAM *et al.* [2]. Samples of the reaction mixture were incubated at 37°C for different periods, and extracted with phenol. The phenol was removed by diethyl ether and the samples (50 μ l, 10⁴-2 $\times$ 10⁴ cpm) were placed on a 5-20% neutral linear sucrose gradient. The material was centrifuged in a SW 56 rotor for 90-120 min (depending on the average molecular weight of the DNA) at 300,000 g at 1°C in a Beckman L 265 B ultracentrifuge and the radioactivity of the collected samples was measured.

Measurement of radioactivity. Samples were placed in scintillation cuvettes containing 5 ml distilled water and activity was detected on the basis of the Cerenkov effect in a Packard Tri-carb type liquid scintillation spectrometer.

Determination of protein and DNA. The method of LOWRY *et al.* [7] was used for estimation of the protein content. The quantity of DNA was measured on the basis of absorption at 260 nm in a Spectromom 204 photometer (1.0 OD 260 nm unit = 47 μ g DNA).

Anti-HEp serum and IgG were prepared by immunizing rabbits with the homogenate of 10⁷ HEP-2 cells and Freund's adjuvant twice weekly over a 4-week period. To obtain the IgG fraction, the serum was purified by anion exchange chromatography on a DEAE Sephadex A 50 column, using as eluent 0-0.5 M NaCl gradient in 0.05 M phosphate buffer (pH 8.1). The preparations were identified by immunoelectrophoresis and fractions in which only IgG was detectable with anti-rabbit horse serum, were used in the following. The IgG preparation did not show exo or endonuclease activity before use.

Results

First, we studied the presence of endonuclease in cell extracts. The virus endonuclease was assumed to decompose the DNA to oligonucleotides and, as compared with the control tissue culture, the increased quantity of acid-soluble material released from the labelled DNA referred to the effect of the virus nuclease. Fig. 1 shows that a significant DNase activity was present in the super-

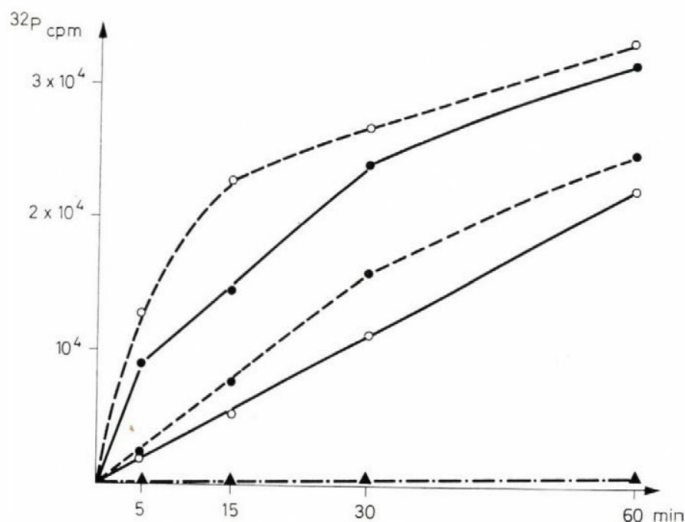


Fig. 1. DNase activity of HEp-2 cell extracts. Amount of acid soluble ^{32}P -labelled nucleic acid fragments is indicated on the vertical axis and the reaction time on the horizontal axis. After incubation at 37°C the quantity of acid soluble labelled material was determined at each interval indicated. ▲—▲ ^{32}P DNA + buffer (0.9% NaCl, 0.001 M phosphate buffer pH 7.2), ○—○ ^{32}P DNA + supernatant of uninfected cell extract; ●—● ^{32}P DNA + supernatant of infected cell extract; ●—● ^{32}P DNA + sediment suspension of uninfected cell extract; ○—○ ^{32}P DNA + sediment suspension of infected cell extract

natant of both infected and control cell cultures as well as in the sediment obtained on centrifugation at 100,000 g. The activity was highest in the control. Spontaneous decomposition of the DNA preparations was insignificant.

Nuclease activity of the cell extracts was estimated on the basis of the sedimentation rate of the DNA substrate in the ultracentrifuge. [As shown in Fig. 2, the supernatant of both infected and control cell cultures decomposed the labelled substrate mainly to low molecular weight fragments. The nuclease activity of the sediment obtained by ultracentrifugation from cell extracts differed slightly. The end products of labelled DNA were of low molecular weight, while DNA fragments of higher molecular weight and faster sedimentation were detected when the DNA was incubated with the sediment of infected cell extracts.

These experiments failed to prove convincingly whether or not a virus-specific nuclease existed in the cells infected with type 1 adenovirus. Since tissue nuclease was measured together with the supposed viral nuclease activity, it was assumed that the tissular enzyme activity covered the viral enzyme activity. Therefore it was tried to demonstrate the enzyme in purified virion preparations. Uninfected tissues were subjected to the same purification

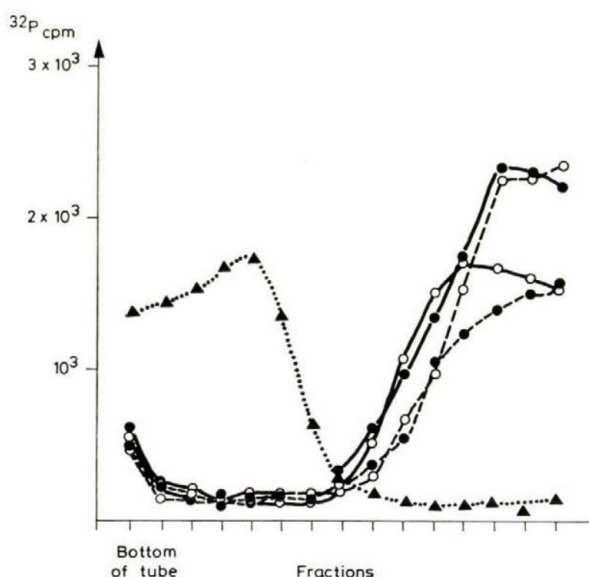


Fig. 2. Effect of HEP-2 cell extract on ^{32}P DNA sedimentation. After incubation at 37°C for 60 min the reaction mixture was extracted by buffer-saturated phenol. The phenol was removed by diethyl-ether and samples were layered on sucrose gradient (5–20% 1.0 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.2). After centrifugation at 300 000 g at 1°C for 90 min fractions were collected and measured for radioactivity. $\blacktriangle$ — ^{32}P DNA + buffer; $\circ$ — ^{32}P DNA + supernatant of uninfected cell extract; $\bullet$ — ^{32}P DNA + supernatant of infected cell extract; $\bullet$ — ^{32}P DNA + sediment of uninfected cell extract; $\circ$ — ^{32}P DNA + sediment of infected cell extract

procedure as the virions and fractions of the density known to contain the virions, were collected with ultracentrifugation. Nuclease activity was subsequently determined in the preparations. No significant acid-soluble material was released under the effect of neither the control nor the virion fractions. Actually it failed to exceed even after incubation at 37°C for 120 min 1% of the ^{32}P labelled DNA content of the reaction mixture.

Nucleic acid analysis performed with ultracentrifugation is presented in Fig. 3. As compared with the untreated DNA, the sedimentation rate of the ^{32}P DNA treated with control fractions did not change after incubation. Nor did incubation with virions for 0 min alter the ultracentrifugation profile. As

shown in Fig. 3, the DNA appeared in fractions of lower density after incubation with virions for 60 min. This indicated that fragments of lower molecular weight developed from the ^{32}P DNA under the effect of virions. This might be explained by the enzyme effect associated with the virion.

It was assumed that in tissues of low nuclease activity the virus nuclease might be demonstrated in the cell extracts, too. Amnion primary monolayer cell cultures were prepared from fresh placenta. Half of the tissues was infected

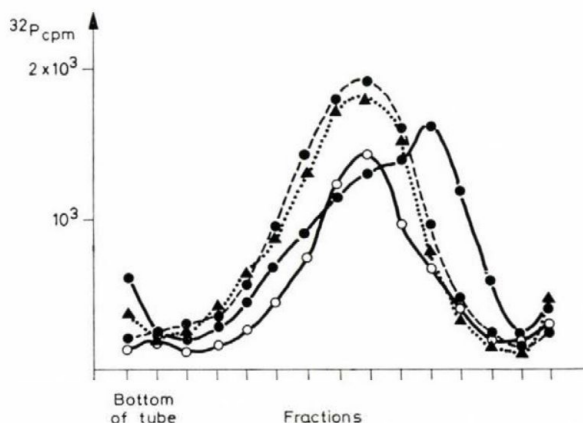


Fig. 3. Effect of purified virions on DNA sedimentation. After incubation the DNA was extracted from the reaction mixture, ultracentrifuged in sucrose gradient, and the collected fractions were measured for radioactivity. Δ ----- Δ ^{32}P DNA + buffer 0 min incubation; $\bullet$ ----- $\bullet$ ^{32}P DNA + purified control, incubation at 37°C for 60 min; $\circ$ ----- $\circ$ ^{32}P DNA + $10\text{ }\mu\text{g}$ of purified virion 0 min incubation; $\bullet$ ----- $\bullet$ ^{32}P DNA + $10\text{ }\mu\text{g}$ of purified virion, incubation at 37°C for 60 min

with 100 TCID_{50} of virus and the other half was left uninfected. Cell extracts were prepared after 10-day incubation both from the infected cells and from the controls. Both extracts released a small amount (less than 1%) of acid-soluble material from the ^{32}P DNA, after 60 min incubation at 37°C . Ultracentrifugation analysis of the DNA substrate treated with amnion extracts showed (Fig. 4) that DNA fractions of lower molecular weight developed under the effect of infected cell extract, whereas the sedimentation of the control extract-treated DNA corresponded to that of the untreated DNA.

These experiments proved the presence of virus-associated nuclease, but the possibility had to be considered that endonuclease molecules of host cell origin might absorb to the virion surface, and exert this activity. To approach this question rabbits were immunized with HEP-2 cells, and IgG not exerting nuclease activity was prepared from the immune serum. As shown in Fig. 5, the sedimentation of DNA was affected in the same way by the untreated virion and by that treated with anti-HEP-2 IgG. This referred to that the anti-HEP-2 IgG failed to inhibit the virion's effect on DNA.

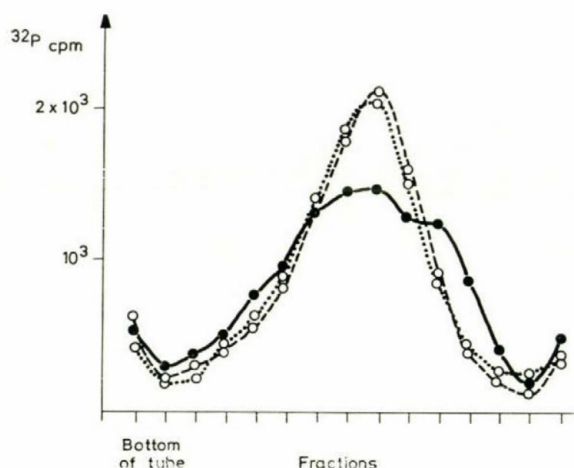


Fig. 4. Effect of amnion cell culture extract on DNA sedimentation. After incubation at 37°C for 60 min the DNA was extracted from the reaction mixture, ultracentrifuged in sucrose gradient and measured for radioactivity. ○·····○ ^{32}P DNA + buffer; ○---○ ^{32}P DNA + uninfected cell extract; ●—● ^{32}P DNA + infected cell extract

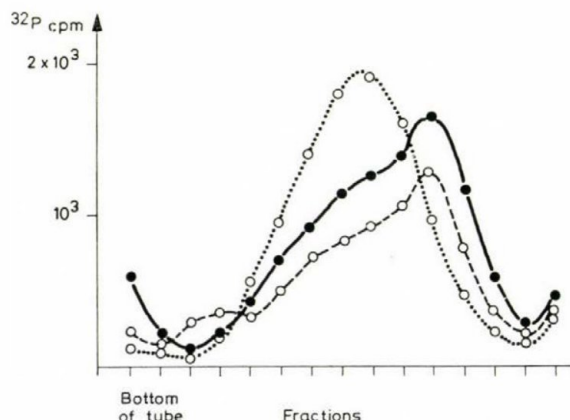


Fig. 5. Effect of anti-HEp-2 IgG and trypsin on virion endonuclease. Virions were pretreated with anti-HEp IgG and trypsin (0.2 mg/ml) at 37°C for 30 min. ^{32}P DNA was added to the mixtures and trypsin inhibitor to the trypsin-containing tubes. The samples were incubated at 37°C for further 60 min. Phenol extraction followed and ^{32}P DNA was estimated by sucrose gradient ultracentrifugation. ●—● ^{32}P DNA + virion; ○---○ ^{32}P DNA + virion + anti-HEP IgG; ○·····○ ^{32}P DNA + trypsin-treated virion

As known from literary data, of the virion capsomeres, only the penton base was found to be trypsin sensitive [8]. According to BURLINGHAM *et al.* [2] the penton endonuclease is inactivated by trypsin; thus we assumed that trypsin inhibited the endonuclease activity of the virion. As shown in Fig. 5, the sedimentation rate of labelled DNA did not decrease under the effect of trypsin-treated virions; this pointed to a loss of the nuclease activity in the virions.

Discussion

BURLINGHAM and DOERFLER demonstrated a new endonuclease activity in KB and hamster embryo cell extracts infected with type 2 and 12 adenovirus. Our experiments suggested that cell extracts of different tissues were not equally suitable for the demonstration of the adenovirus-associated endonuclease. Owing to the fact that different host cell nucleases were present in these extracts, though the individual cellular nucleases were not studied separately, it was assumed that these enzymes affected also the substrate added to the cell extract. This might serve to explain the fact that while the adenovirus-associated endonuclease was successfully demonstrated in primary amnion cell cultures of low nuclease activity, it failed to be detectable in HEp-2 tissue extracts. Endonuclease activity was demonstrated in our experiments in purified type 1 adenovirion, similarly to that described with serotype 2 and 12 [1]. Literary data as well as our own findings suggest that of the virion's surface components, it is the trypsin sensitive penton that is associated with the endonuclease activity. This assumption needs, however, further elucidation. At the same time, a host cell endonuclease attaching to the virion or penton surface might also exert such an activity. Our observation that the host cell antiserum was unable to inhibit the virion endonuclease, referred to the virion origin. The endonuclease-inhibiting effect of antiviral immune sera is not a final proof in this problem [1, 2], as the virus-associated antibodies precipitate not only the virion, but also the cellular nucleases which are supposed to adsorb to the surface, thus the enzyme is no more able to attach to the substrate molecules.

The significance of the adenovirus endonuclease is not yet known in the host cell-virus relation. Nor is it known, whether the host cell DNA or the virus DNA is the substrate of the enzyme *in vivo*, and it is unclear whether the enzyme is coded by the adenovirus genome or it appears as an induced enzyme in the cell.

*

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VIRUS CONTENT AND EFFECTIVENESS OF FOOT-AND-MOUTH DISEASE VACCINES*

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Summary. (i) The amount of 22 nm particles in 26 batches of cattle tongue epithelium extract used for the preparation of C-type vaccine was determined with an improved 50% haemolytic and point complement fixation test after fluorocarbon precipitation of non-immunizing 7 nm particles. The total amount of 22 nm and 7 nm particles (α GN value) varied considerably from batch to batch, 22 nm components (α GF value) showing a maximum 5-fold difference. (ii) Effectiveness of vaccines with known virus content was tested in adult mice challenged with an adapted virus strain. In commercial C-type vaccines the complement-fixing activity of 22 nm particles and the potency of the vaccine showed a logarithmic regression (mouse index = $-1.43 + 2.27 \lg \alpha$ GF).

Of the different structural units of foot-and-mouth disease virus only the 22 nm component (140S and 75S) is immunogenic; 7 nm particles (14S) have no such activity. For the activity, the 140S complete nucleocapside and the 37S RNA are responsible [–5]. Accordingly, the amount of 22 nm particles is of decisive importance in vaccine preparation.

The properties and mode of action of organic solvents used for virus purification and for the separation of components has been summarized by PHILIPSON *et al.* [6]. The introduction of fluorocarbons [7] made many of these substances dispensable. Fluorocarbons dissolve lipids [8], denature large and moderately large protein molecules but do not affect small or medium ones [9, 10], nor virus-sized nucleoproteins [11]. In view of these properties, fluorocarbons are advantageously used for the purification of different viruses [8, 12–15], all the more so as the complete virion and its various components highly differ in sensitivity to these compounds [8–10].

Non-viral proteins and 7 nm virus particles are removed by precipitation from foot-and-mouth disease virus extract and 22 nm (140S and 75S) particles remain in the preparation [16, 17]. The amount of the effective antigen component in the purified vaccine can be determined by complement fixation reaction [18].

The purpose of the present work was to determine the complete virus and virus component content of lingual epithelium used for vaccine production, and to elucidate the association between the amount of immunologically active virus particles and the effectiveness of vaccines prepared from them.

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Materials and methods

Virus Type C virus was produced in the tongue epithelium of cattle *in vivo* and stored at -40°C . The extract was prepared by adding 10 volumes of 0.01 M glycine buffer (pH 7.6) to 1 volume virus, spinning in a flow-centrifuge twice at 16,000 rpm and filtering through Schleicher-Schüll Selectron 1118 membrane laminates. The extract prepared in this manner for vaccine production was sampled for the experiments.

Purification of virus. Trichlorotrifluoro ethane (Arcton 113, Serva) found best for this purpose was used; the compound's solubility in water is <0.05 at 25°C [6]. To 1 volume Arcton 113 2 volumes of virus extract were added and the mixture was shaken vigorously for 15–20 min. Undesirable proteins precipitated at the border of the two phases as a gelatinous substance; the virus was collected by harvesting the supernatant. The procedure was repeated until the gelatinous substance failed to appear at the phase border (usually 3–4 times). After purification the complement-fixing activity of the virus suspension became constant.

Complement-fixing activity was determined by the quantitative test described by WADSWORTH *et al.* [19] and BRADISH *et al.* [20] as modified by one of us. The complement was standardized so that 7 μl aliquots gave 50% haemolysis. Complement-fixing activity of virus samples was expressed as μl of standardized complement fixed in the excess of antibody (αG). By calculating the ratio of complement-fixing activity for fluorocarbon-treated samples (αGF) and for untreated samples (αG), the absolute and relative amount of 22 nm particles was determined [21].

Vaccine production. The filtered virus suspension was adsorbed to aluminium hydroxide gel and inactivated with formalin at 26°C for 41 hr. Final concentrations of these substances were 1.2% (Al_2O_3 and 0.04% (formaldehyde), respectively.

Mouse index. The effectiveness of vaccines with known virus content was determined by the mouse index method. An F_1 population of 8-week-old mice weighing 17–25 g bred from Balb/c ♀ and CBA ♂ mice was used. The degree of effectiveness was estimated from the logarithm of the ratio of titres for non-vaccinated and vaccinated animals challenged with the virus strain adapted in 10–15 passages to adult mice. Titration was performed two weeks after vaccination by injecting tenfold serial dilutions of the virus to groups of 10 mice.

Results

Association between the purified virus content and effectiveness of 26 batches of foot-and-mouth disease vaccine comprising a total of about 3 million doses was examined. One dose (2 ml) of vaccine represented the virus content of 0.13 g lingual epithelium and contained 140S, 75S, 14S and 37S components together. Treatment with fluorocarbon removed 14S (7 nm) particles, 37S RNA had no complement-fixing activity, and, thus, the titre expressed the amount of 22 nm particles. Results obtained before and after fluorocarbon treatment are presented in Table I.

Table I

Amount of C-type foot-and-mouth disease virus components in one dose (2 ml) of vaccine
Number of batches tested, 26

N 22 + 7 nm			F 22 nm			F/N		
$\bar{N}$	s_N	CV	$\bar{F}$	s_F	CV	$\bar{F}/\bar{N}$	$s_{F/N}$	CV
286	± 85	29%	187	± 70	37%	0.64	± 0.14	22%

$\bar{N}$ = complement-fixing activity of untreated virus samples

$\bar{F}$ = complement-fixing activity of fluorocarbon-treated virus samples

CV = coefficient of variation

s = standard error

N values (total amount of 22 nm and 7 nm particles) and F values (amount of 22 nm particles) thus the actual virus content of individual lingual epithelium samples, varied considerably (CV 29% and 37%, respectively). F/N values expressing the relative amount of the components varied accordingly (CV 22%).

The $\overline{F/N} = 0.64 \pm 0.14$ value found in the present study is higher than that described by MUSSGAY [9] and by BROWN and CARTWRIGHT [10]

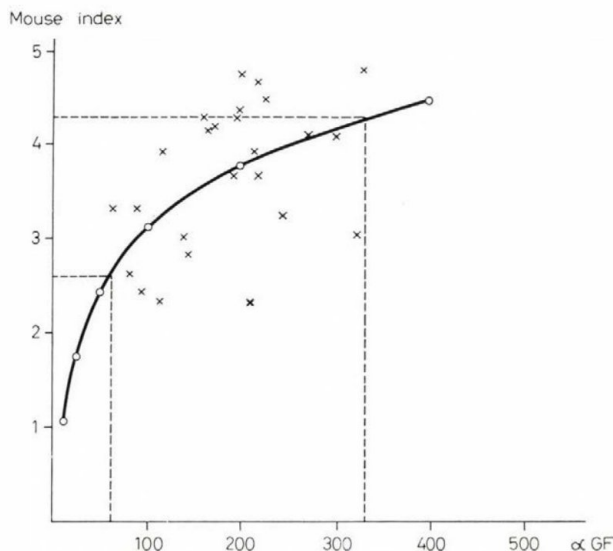


Fig. 1. Logarithmic regression between α GF and mouse index values

for tissue virus, but stands close to the value shown by MACKOWIAK *et al.* [4] for Fraenkel virus.

The highest difference was 4 to 5-fold between vaccines prepared from identical amounts of lingual epithelium. Thus, standardization of vaccines to uniform effective virus antigen content can be accomplished only if the F and F/N values of the virus suspensions are known.

In subsequent experiments the association between the amount of active components 140S and 75S [1] and the protective effect was examined. The amount of 22 nm particles in different batches of vaccine was determined and expressed in complement-fixing activity (α GF). The potency was estimated in mice (mouse index). The values for the two parameters were plotted as shown in Fig. 1. The logarithms of α GF values and mouse index showed a linear regression (Fig. 2). The equation for the regression can be expressed by the general formula $Y' = a + b \lg X$ as follows:

$$\text{Mouse index} = -1.43 + 2.27 \lg \alpha \text{GF}$$

The regression between the parameters was significant by the F test in case of $FG = 1$ and $n-2$ ($n = 26$) at the level of $P = 5\%$.

Limits of confidence, based on $FG = n-2$, $t_{P=5\%}$, are shown in Fig. 2.

The virus content of different batches of vaccine ranged between 60 and 320 ($\alpha GF = 187$). The part of the regression curve falling in this interval is marked in Fig. 1 in order to distinguish it from extrapolated parts.

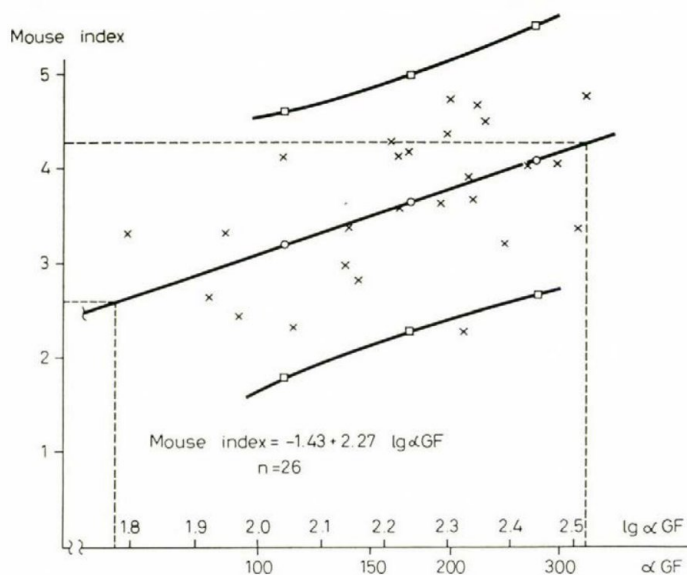


Fig. 2. Linear regression and confidence limits between $\lg \alpha GF$ and mouse index

It is evident that, if the amount of virus antigen is increased gradually, the mouse index increases less and less. This means that antigen doses higher than the optimum for an effective immunity, are superfluous. The examined range of αGF falls between mouse index values 2.6–4.3. As examined with the Hungarian Standard Potency Assay method in cattle, all batches of vaccine studied in this work were satisfactory. This finding may, on the one hand, be useful for the evaluation of the above-mentioned mouse index range; on the other hand it shows that immunological consequences of alteration over a given degree of the amount of virus antigen remain undetected with the standard assay procedure.

Discussion

Cattle lingual epithelium preparations varied greatly [60–320 αGF] in virus content and, accordingly, the F and F/N values should be considered when standardizing vaccine. Knowledge of the regression between virus

content per dose and potency allows to determine the optimum amount of antigen. By standardizing vaccines in this manner, their uniform protective effect can be guaranteed. The present studies have confirmed the hypotheses and findings of different investigators as to the association between the amount of virus antigen and potency of the vaccine [2, 3, 5, 22, 23].

The mouse index values of vaccines with known virus content extrapolated from the regression equation, ranged between 2.6 and 4.3. As all batches of vaccines were satisfactory in the standard potency test in cattle, conclusions may be drawn for the value of the method described in this paper. The mouse index test is advantageous partly in view of the fact that, using a great number of mice, it allows an economic statistical evaluation, and partly because it reveals differences between vaccines remaining undetected with the standard assay procedure.

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ANTIGENIC RELATIONSHIP BETWEEN MORGANELLA MORGANII AND YERSINIA ENTEROCOLITICA

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Summary. Two new O antigens have been described for the *Morganella morganii* antigenic schema. O antigens of the strains representing the new serotypes (O43 : H2 and O44 : H19, 37) are related to *Yersinia enterocolitica* O9 and O17, respectively.

Human yersinia infections seem to have an increasing importance in Hungary. Country-wide isolations of *Yersinia enterocolitica* from cases of enteritis and appendicitis, from outbreaks among children and from the environment amounted to 1200 strains during the period of this study. Microbiological and epidemiological characteristics of the agent have been described by various authors [1-5]. Less data are available for the antigenic relationships of *Y. enterocolitica*.

Serological relationships within *Enterobacteriaceae* are well-known and intra- and interfamilial relationships of *Morganella morganii* have been examined by a number of workers [6-12]. As to *Y. enterocolitica*, WINBLAD mentioned that sera prepared with heated cultures reacted weakly with *Proteus*, while after immunization with living suspensions no such antibodies appeared [13]. In this paper we described antigenic relationships between *M. morganii* and *Y. enterocolitica*.

Materials and methods

Strains. Two *M. morganii* strains designated Louvain and Belgian were received from Professor G. WAUTERS (Louvain, Belgium). *Y. enterocolitica* type strain 383 (O9) and 955 (O17) were supplied by the National Institute of Public Health, Budapest.

Biochemical reactions were performed as described in reference [14].

Serological examinations were made with living and heated (2 1/2 hr, 100°C) agar slant cultures incubated at 37°C. H antigens were prepared by passing the cultures through U tubes at 22°C, subculturing on soft agar plates then in broth, and preserving with 0.5% formalin. Tube agglutination was read after incubation at 37°C for 24 hr. Agglutinin absorption was carried out in the usual manner.

Results

The two *M. morganii* strains were received from Belgium in order to include them in the antigenic schema and to examine their relationship to *Y. enterocolitica*. Both strains were biochemically typical and belonged to biotype 1 of *M. morganii* [15].

O antigens. In O sera for the known 42 groups of *M. morganii* the isolates failed to agglutinate. With O serum prepared against strain Belgian a unilateral relationship was shown to *M. morganii* O5 and O25. As no other serological connections were revealed, strain Louvain was chosen to represent antigen O43 and strain Belgian was designated as type strain for antigen O44.

Table I
H antigen of M. morganii strain Louvain

H antigen	H serum for type strain H2		H serum for strain Louvain	
	Unabsorbed	Absorbed by Louvain H	Unabsorbed	Absorbed by type strain H2
Type strain H2	25 600	—	12 800	—
Louvain	12 800	—	6 400	—
H antigen	2		2	

H antigens of strain Louvain. Table I shows that the strain agglutinated in serum H2 practically in full titre and *vice versa*. After cross-absorption, low titre O-type agglutinations appeared, which were attributable to O agglutinins. Absorption of O agglutinins was not necessary, since H and O-type reactions were easily distinguishable, the strains were not related in somatic antigens and the titre of H antibodies was high. Accordingly, the flagellar antigen of strain Louvain was identical with that of type strain H2.

H antigen of strain Belgian. In H antigens *M. morganii* type strain O29 : H19 and strain Belgian were bilaterally related. The relationship was

Table II
H antigens of M. morganii strain Belgian

H antigen	H serum for type strain H19		H serum for strain Belgian	
	Unabsorbed	Absorbed by Belgian H	Unabsorbed	Absorbed by type strain H19
Type strain H19	12 800	1600	1600	—
Belgian	3200	—	6400	6400
H antigens	19.38	38	19.37	37

of the a, b-a, c-type: the two cultures had antigen H19 in common and strain Belgian contained partial antigen H37 in addition. Antigen H19, described previously as a single factor was, accordingly, shown to consist of two partial antigens in the type strain (H19 and H38).

Serological relationship between the isolates and Y. enterocolitica. In order to exclude the presence of *Y. enterocolitica* antibodies as a result of natural infection, the rabbits were sampled before immunization and their sera were tested by agglutination.

Table III

Antigenic relationship between the O antigens of M. morganii 044 and Y. enterocolitica 017

O antigens	O serum for strain Belgian		Serum <i>Y. enterocolitica</i> 017	
	Unabsorbed	Absorbed by <i>Y. enterocolitica</i> 017 100°C	Unabsorbed	Absorbed by Belgian 100°C
Belgian 100°C	2560	2560	160	—
<i>Y. enterocolitica</i> O 17, 100°C	640	—	5120	640
Antigenic relationship	a, b	b	a, c	c

Cross-agglutination and cross-absorption experiments with strain Belgian are shown in Table III. In O serum for strain Belgian, *Y. enterocolitica* 017 gave a low titre agglutination; absorption of serum Belgian with *Y. enterocolitica* 017 failed to decrease the agglutinin titre for the former.

Although in serum *Y. enterocolitica* 017 strain Belgian gave a relatively low titre agglutination, after absorption it considerably decreased the homologous titre. Accordingly, the a, b-a, c relationship between the two organisms is characterized by a less marked common factor and a well-developed specific factor in strain Belgian and by a predominating common factor in *Y. enterocolitica* 017. Cross-absorption with living cultures failed to decrease the homologous agglutinin content of the sera.

As shown in Table IV, strain Louvain and *Y. enterocolitica* 09, too, exhibited an a, b-a, c relationship.

Table IV

Antigenic relationship between the O antigens of M. morganii 043 and Y. enterocolitica 09

O antigens	O serum for strain Louvain		Serum <i>Y. enterocolitica</i> 09	
	Unabsorbed	Absorbed by <i>Y. enterocolitica</i> 09, 100°C	Unabsorbed	Absorbed by Louvain, 100°C
Louvain, 100°C	5120	1280	640	—
<i>Y. enterocolitica</i> O 9, 100°C	640	—	2560	2560
Antigenic relationship	a, b	b	a, c	c

Subsequently, sera were produced with living strains Belgian and *Y. enterocolitica* O17. In these sera the heterologous strain reacted at low titre. Absorption with living or heated heterologous antigens failed to decrease the homologous titres; in this manner no antigenic relationship was demonstrable. These data are in agreement with WINBLAD's observation [13].

Discussion

The serological relationship revealed between *Y. enterocolitica* and other enteric bacteria support the view that this species belongs to the family *Enterobacteriaceae*. As in Europe *Y. enterocolitica* serogroups O9 and O3 are predominant, their antigenic relationships to other bacteria may cause diagnostic problems. In view of an antigenic relationship between *Y. enterocolitica* O9 and *Brucella* [16], patient sera giving positive agglutination with the former, should be checked with *Brucella* antigen. BARUA and WATANABE [17] described a unilateral connection between *Y. enterocolitica* and *Vibrio cholerae*. LYSY and KNAPP [18] showed that patient sera agglutinating OH suspensions of *Y. enterocolitica* can partially or completely be absorbed with *M. morganii* OH antigen but not with corresponding heated antigen. In contrast, we found that antigenic relationship between the two species can be detected with heated suspensions and not with living cultures.

From the results of the present examinations it may be concluded that patient sera agglutinating *Y. enterocolitica* O9 or O17 heated suspensions should be checked with *M. morganii* O43 and O44 antigens and, in the case of positive reaction, the sera should be absorbed with the corresponding *M. morganii* strain and re-examined with *Y. enterocolitica*. New isolates agglutinating in *Y. enterocolitica* O9 and O17 sera should be examined in *M. morganii* O43 and O44 sera, respectively. Further examinations are required to characterize other antigenic relationships between the two species.

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REISOLATION AND CHARACTERIZATION OF HAEMOPHILUS INFLUENZAE-MURIUM

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Summary. Twenty-three *Haemophilus influenzae-murium* strains were isolated from the nasopharynx of healthy CFLP white mice. Microbiological characterization of the cultures confirmed the taxonomic position of the organism which had become problematic due to the loss of the original isolates. The type strain proposed, EO₁, is available from the Hungarian National Collection of Medical Bacteria, National Institute of Public Health, Budapest as 95001.

The genus *Haemophilus* contains several species widely distributed in man and animals [1]. Human strains have been studied in detail, whereas the classification of animal isolates still presents problems.

Haemophilus influenzae-murium was first identified by KAIRIES and SCHWARTZER [2] who isolated it as a causative agent of epizootic respiratory infection of mice in 1935. IVÁNOVICS and IVÁNOVICS [3] isolated the organism in 1937 and described its growth factor requirement.

From a report on the taxonomy of *Haemophilus* to the International Committee on Nomenclature of Bacteria, it appeared that the original isolates of *H. influenzae-murium* had been lost and thus the organism's classification has become problematic [4].

In November and December, 1973, throat and nose specimens were cultured in this institute from 200 healthy CFLP white mice. The examinations were performed using groups of 50 mice at four different occasions. In one of the groups, 23 mice harboured an organism showing the characteristics of *H. influenzae-murium*.

Material and methods

Cultural methods. The mice were killed by ether. The pharyngeal cavity was swabbed and streaked on chocolate agar and the nasal mucosa was removed and placed into Levinthal broth. The broth cultures were incubated at 37°C for 6 hr then subcultures were incubated at 37°C for 6 hr then subcultured on chocolate agar.

Culture media. Nutrient agar was prepared from beef infusion with 2% agar and 1% Witte peptone (pH 7.4). To make chocolate agar, nutrient agar at 80°C was supplemented with 5% ox blood and the bottles were left to stand at the same temperature for 30 min. Levinthal agar was prepared by filtering chocolate agar through cotton wool. For the enrichment of

chocolate agar, 250 g baker's yeast were boiled for a few minutes in 750 ml distilled water; on the next day the supernatant was passed through Seitz filter. Levinthal broth was made by heating 5% ox blood in broth at 80°C for 30 min and Seitz filtering.

Growth factor requirement. (1) From 24 hr Levinthal broth cultures dilutions ranging from 10^{-1} to 10^{-6} were prepared and 0.05 ml aliquots were streaked with glass rods on agar, blood agar (5% ox, rabbit or human blood), chocolate agar with or without 10% yeast extract. The number of colonies and their ability to grow in subculture were examined after incubation at 37°C for 24 hr. (2) Agar medium containing 5% and 10% ox serum, 5% and 10% ox blood, nutrient agar and chocolate agar were seeded with saline suspensions (one loopful of bacteria mixed in 1 ml physiological saline) and growth characteristics were compared. (3) Haematin prepared from ox blood as described by BUTLER [5] was added at different dilutions to nutrient agar medium. Yeast agar plates were prepared with 2.5, 5 and 10% yeast extract. The plates were inoculated with saline suspensions. (4) Haematin (National Biochemical Corporation, Cleveland) and DPN (Reanal, Budapest) were added at gradual dilutions to nutrient agar. Growth factor requirement was examined by seeding the plates with saline suspensions.

Biochemical tests. Indole, nitrate, H_2S , methyl red, Voges-Proskauer reactions and carbohydrate breakdown were tested in Levinthal broth. Indole production was tested with Kovács's reagent after 24 hr, the Voges-Proskauer reaction with Barrit's reagent after 3 days; the methyl red and nitrate tests were performed in 3-day cultures [6]. H_2S production was detected with lead acetate paper strips and acid production from carbohydrates with bromthymol blue indicator; observation lasted for 14 days. The following biochemical tests were carried out with rapid methods. (a) Urease activity: to 2% aqueous urea (pH 7.6) 0.5% phenol red stock solution (0.02%) was added; one ml solution was seeded with a loopful of bacteria; an urea-free medium was used as control [7]. (b) ONGP was dissolved and filtered; one half ml solution was inoculated with a loopful of bacteria and readings were made after 2 and 4 hr [8]. (c) Tryptophan and phenylalanine deaminase tests: one per cent amino acid was dissolved in distilled water and 0.5 ml aliquots were seeded with one loopful of culture; after 4 hr incubation 0.1 ml ferric chloride reagent was added [6].

Antibiotic sensitivity was tested by the use of susceptibility disks (Institute for Serobacteriological Production and Research Human, Budapest) applied on 10% ox blood agar plates swabbed densely with saline suspensions.

Pathogenicity experiments. Seronegative mice and guinea pigs not harbouring *H. influenzae-murium* on their nasopharynx were chosen. For infection, saline suspension of strain EO₁ (10^9 cells/ml) harvested from 24 hr chocolate agar plates, was used. Doses of 0.2 or 0.5 ml were given to mice and 0.5 or 1 ml to guinea pigs intraperitoneally. Intranasal infection was carried out under general ether anaesthesia; the doses were 0.2 ml for mice and 0.5 or 1 ml for guinea pigs. After intranasal infection, part of the animals was kept at 4°C for 2 hr.

Serological reactions. Seronegative rabbits not harbouring *H. influenzae-murium* in their nasopharynx were immunized with living culture EO₁. Tube agglutination test in serum EO₁ was performed with living suspensions of 22 *H. influenzae-murium* strains and *H. influenzae* type a (a/4428), type b (m/4118), type c (a/1060), type d (w/4044), type e₁e₂ (p/0323), type f(s/9464) strains. Twenty-five *H. influenzae* strains isolated in this institute from patients with sinusitis, otitis and meningitis, and 40 *H. parainfluenzae* strains cultured from the throat of healthy persons, were also examined for agglutination in serum EO₁.

Results

The following characteristics of *H. influenzae-murium* are described on the basis of studies on 23 isolates. Strain EO₁ designated as type strain for the species is available from the Hungarian National Collection of Medical Bacteria, National Institute of Public Health, Budapest as 95001.

Morphology. Gram-negative coccobacilli or pleomorphic rods; encapsulated, non-motile at 22°C or 37°C.

Cultural characteristics. No growth on nutrient agar; on blood agar high convex, slimy colonies 1–2 mm in diameter; on chocolate agar somewhat larger, more slimy colonies; on 5% egg yolk agar yellowish colonies 1–2 mm in diameter; fails to grow on Endo or Löffler media.

Levinthal broth cultures are characterized by a deposit and slight turbidity. Growth occurs in egg broth but not in simple broth. Multiplication is inhibited under anaerobic conditions and markedly hindered in the presence of 10% CO₂.

Growth factor requirement. All strains required X factor for growth; V factor *per se* failed to support multiplication. In combination with X factor, however, V factor stimulated the production of slime substance.

(1) On nutrient agar plates seeded directly with 24 hr Levinthal broth, minute colonies (200 per 720 viable cells inoculum) developed; these colonies failed to grow on subculturing.

Blood agar plates prepared with either ox, rabbit or human blood, supported the growth well. After seeding from 24 hr Levinthal broth, 720 colonies developed on blood agar from an inoculum containing the same number of viable cells recovered on chocolate agar. The growth was transferable to fresh blood agar.

If chocolate agar was supplemented with 10% yeast extract, the colonies became larger (4–5 mm in diameter) and more slimy. A similar enhancement of growth was observed if the agar's ox blood content was raised from 5% to 10%.

(2) On agar containing 5% or 10% serum the growth was similar to that observed in simple agar.

(3) If added to 100 volumes of nutrient agar, one volume of haematin prepared by BUTLER's technique allowed the same degree of growth as 10% ox blood agar. Few colonies developed on simple agar containing 10% yeast extract.

(4) N. B. C. haematin, when added at 80–100 µg/ml to nutrient agar, yielded the same number of colonies and the same colony forms as did 10% ox blood agar plates. DPN *per se* failed to support growth, but in combination with haematin it increased slime production.

Biochemical reactions. The 23 isolates behaved uniformly (Table I). Features characteristic of the strains were nitrate reduction, failure to produce indole, urease and H₂S, acid production in glucose, maltose, sucrose and fructose but not in lactose and mannitol.

The antibiotic sensitivity spectrum of the isolates is presented in Table II.

Pathogenicity. After intraperitoneal infection the mice showed no symptoms of illness and produced no detectable antibodies against the agent. Intranasal infection resulted in pyrexia and conjunctivitis; none of the animals died. In one animal the periocular tissue became swollen and an abscess developed, from which the agent was recovered. By the end of the 21-day observation period, many of the animals had become seropositive and harboured the organism in their throat (Table III).

Table I

Characteristics of 23 H. influenzae-murium isolates

X factor	+	Arabinose	—
V factor	—	Xylose	—
Serum	—	Rhamnose	—
CO ₂	—	Glucose	+
Haemolysis	—	Fructose	+
Motility, 22 and 37°C	—	Galactose	—
Capsule	+	Mannose	+
Sporulation	—	Lactose	—
Optimal growth, °C	37	Maltose	+
Endo agar	—	Sucrose	+
Löffler medium	—	Trehalose	+
Egg medium	+	Raffinose	—
Growth with 6.5% NaCl*	—	Inulin	—
Gelatin hydrolysis	—	Starchn	—
Litmus milk	—	Aesculi	—
Indole	—	Salicin	—
Urea	—	Adonitol	—
Nitrate to nitrate	+	Dulcitol	—
H ₂ S	—	Inositol	—
Methyl red	—	Mannitol	—
Voges-Proskauer	—	Sorbitol	—
Oxidase	—	OF test	—
Catalase	+	ONPG	±
Phenylalanine deaminase	—		
Tryptophan deaminase	—		

* Tested on blood agar and chocolate agar containing 6.5% NaCl

None of the 6 intraperitoneally and 4 intranasally infected guinea pigs became ill, produced antibodies or harboured the organism. Exposure to 4°C after infections also failed to produce signs of illness.

Serological examination showed that the isolates were related antigenically. According to agglutination titre in serum EO₁, the isolates were distributed as follows: strain EO₁ 1 : 1280, 3 strains 1 : 640, 11 strains 1 : 320, 7 strains 1 : 160 and 1 strain 1 : 80. None of the *H. influenzae* strains tested reacted in serum EO₁; 12 of the 40 *H. parainfluenzae* strains agglutinated at 1 : 20 titre.

Table II
Antibiotic sensitivity of 23 H. influenzae-murium strains

Drug	Amount in disk	No. of strains		
		Sensitive	Moderately sensitive	Resistant
Penicillin	3 IU	16	7	—
Oxacillin	10 µg	—	18	5
Methicillin	20 µg	10	13	—
Ampicillin	20 µg	22	1	—
Carbenicillin	50 µg	23	—	—
Cephalosporin	10 µg	19	4	—
Erythromycin	10 µg	23	—	—
Oleandomycin	30 µg	12	11	—
Novobiocin	30 µg	3	20	—
Vancomycin	50 µg	—	—	23
Lincomycin	10 µg	—	21	2
Pristinamycin	10 µg	23	—	—
Spiramycin	30 µg	6	17	—
Streptomycin	30 µg	—	23	—
Kanamycin	30 µg	7	16	—
Neomycin	100 µg	13	10	—
Gentamicin	20 µg	13	10	—
Paromomycin	50 µg	17	6	—
Chloramphenicol	30 µg	23	—	—
Tetracyclin	30 µg	23	—	—
Polymyxin B	15 µg	23	—	—
Colistin	20 µg	23	—	—
Furadantin	300 µg	23	—	—
Nalidixic acid	30 µg	19	4	—

Table III
Intranasal infection of mice with H. influenzae-murium

Mice used			Findings 21 days after infection	
Source	Strain	No. of animals	No. of sero-positive animals	No. of carrier animals
Our institute	CFLP	10	5	7
	C ₃ H	10	6	5
Institute for Laboratory Animals	CFLP	10	8	7
	C ₃ H	5	—	2
	Balb/c	5	1	4

Discussion

Outbreaks of febrile upper respiratory tract infection and bronchopneumonia in mice associated with small Gram-negative rods were reported by ANDREW *et al.* [9]. HOYLE [10] described coccobacilli growing in translucent colonies only on Levinthal agar as a causative agent of bacterial complication of virus pneumonia in mice. KAIRIES¹ and SCHWARTZER [2] were the first to define haemophilus-like organisms isolated from the respiratory tract of mice. The agent, named *H. influenzae-murium*, caused an outbreak of bronchopneumonia and conjunctivitis, and was present in 35–70% of healthy mice after the outbreak. IVÁNOVICS and [IVÁNOVICS [3] isolated from the pharyngeal and nasal washings of 14 out of 19 normal mice an organism identical in morphological, biochemical and serological characteristics with KAIRIES and SCHWARTZER's bacterium. The organism was pathogenic for mice and required X factor for growth. In Bergey's Manual, on the basis of the above findings, PITTMANN summarized the properties of *H. influenzae-murium* [11]. The characteristics of the original isolates and of our strains are compared in Table IV.

Table IV
Comparison of the characteristics of original and new isolates of H. influenzae-murium

	Original isolates*	New isolates
X factor	+	+
V factor	—	—
Haemolysis	—	—
Motility	—	—
Endo agar	+	—
Egg broth	+	+
Gelatin hydrolysis	—	—
Litmus milk	—	—
Indole	—	—
Optimal growth, °C	37	37
Glucose	+	+
Fructose	+	+
Lactose	+	—
Maltose	+	+
Sucrose	+	+
Mannitol	—	—
Pathogenicity to mice	weak	weak
Pathogenicity to guinea pigs	nil	nil

* KAIRIES and SCHWARTZER [2]

Except two features, our results have confirmed the data of KAIRIES and SCHWARTZER. The discrepancy in growth on Endo medium and in fermentation of lactose may be attributed to the fact that KAIRIES and SCHWARTZER regarded minute red colonies appearing after 2-3 days on Endo agar as evidence of growth and of lactose fermentation. In standardized Levinthal broth our cultures failed to attack lactose and no growth occurred on Endo agar. Still, they gave a weak ONPG reaction; i. e. beta-galactosidase production, even if in small amounts, is characteristic of the species.

In the present work, in addition to 16 characters mentioned in the original description, *H. influenzae-murium* was tested for another 32 reactions. Of these, nitrate positivity, a uniform property of *Haemophilus*, is especially important. The isolates differed from *Pasteurella*, *Brucella* and *Actinobacillus* in their failure to grow on nutrient agar; from *Francisella* in not requiring a special medium; from *Bordetella* in attacking carbohydrates; and from *Moraxella* in being oxidase negative.

On the basis of growth factor requirement, the genus *Haemophilus* may be classified into 3 groups as follows. (1) X+V factor required: *H. influenzae*, *H. aegyptius*, *H. haemolyticus*, *H. gallinarum*, and *H. suis*. (2) V factor required: *H. parainfluenzae*, *H. parahaemolyticus*, *H. paraphrophilus*, *H. parahaemolyticus*, *H. paragallinarum* and *H. parasuis*. (3) X factor required: *H. ducreyi*, *H. aphrophilus*, *H. haemoglobinophilus* and *H. ovis*. *H. citreus*, *H. piscium* and the ill-defined *H. putriorum* are less dependent on X factor.

H. influenzae-murium belongs to the X factor-depending group. BOYCE *et al.* [12] showed that *H. aphrophilus*, varying from strain to strain, split off more or less X factor independent clones. *H. influenzae-murium* isolates examined in the present study showed some rudimentary growth on agar seeded with saline suspension of EO₁ strain. X factor independent variants capable of growing in serial subcultures on agar were, however, not isolated.

H. influenzae-murium can be differentiated from other X factor-requiring species. *H. ducreyi* and *H. aphrophilus* multiply only in the presence of CO₂, whereas the growth of our isolates is markedly inhibited by CO₂. *H. haemoglobinophilus* differs from *H. influenzae-murium* in producing indole and splitting xylose and mannitol, but not maltose. *H. ovis* attacks a broad spectrum of carbohydrates (hexoses, disaccharides, mannitol, sorbitol). *H. citreus*, in contrast to our isolates, is haemolytic and indole positive. *H. piscium* differs from all blood-requiring *Haemophilus* species in being able to grow with diphospho-thiamine.

All *H. influenzae-murium* cultures were resistant to vancomycin. This antibiotic may, accordingly, be used for the selective isolation of the organism, for example, on Füzi's *H. influenzae* medium [13] containing 25 µg vancomycin per ml.

As the organism isolated in this study was encountered in healthy mice, and caused a mild disease only in great intranasal doses resulting in a 50% seropositivity of the animals, in agreement with the opinion of IVÁNOVICS and IVÁNOVICS [3], it should be regarded as an infrequent member of the nasopharyngeal flora of the mouse which, under certain conditions, may become pathogenic. The first epizootic described by KAIRIES and SCHWARTZER [2] took place in the winter and HOYLE [10] isolated a similar organism from mice with virus pneumonia. The fact that our *H. influenzae-murium* strains too were isolated during the winter, suggests that human and mouse haemophilus organisms are similar in seasonal incidence. Accordingly, the presence and potential pathogenicity of *H. influenzae-murium* in mice subjected to immunosuppressive treatment (X-ray irradiation, cytostatic substances, etc.) should be reckoned with.

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MOLECULAR ORDER OF CARBOHYDRATE COMPONENTS IN CELL WALLS OF BACTERIA, FUNGI AND ALGAE ACCORDING TO THE TOPO-OPTICAL REACTION OF THE VICINAL OH GROUPS

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Summary. Selective topo-optical staining of vicinal OH groups with aldehyde-bisulphite-toluidine blue (ABT) has been used for studying the molecular structural order of polysaccharide components in microbial cell walls and capsules. (i) By intensive metachromatic staining in the light microscope, the method demonstrates carbohydrates, containing free vicinal OH or acylaminohydroxyl groups. (ii) The birefringence induced by oriented toluidine blue binding of the ABT reaction, provides information about the linear order of the reacting carbohydrate chains, a possibility not offered by other ultrastructural methods. Furthermore the different optical character of birefringence induced by ABT is indicative of the presence of (a) tangentially oriented polysaccharide chains in the cell wall of the molds and bacterial capsules; or (b) radially oriented OH groups suggesting helical glycan chains of peptidoglucomannane and mucopeptide in the yeasts and bacterial cell walls.

The chemical and structural organization of bacterial and lower plant cell walls is heterogenous even within the same taxonomic group. The structural basis of the bacterial cell walls is formed by a mucopeptide bag associated with other carbohydrate components: in Gram-positive bacteria, teichoic acids; in the outer layer of the cell wall of Gram-negative bacteria, lipopolysaccharides. The heterogeneity of these components in the cell walls is so great that various serological types have been differentiated according to their content of, or lack in different hexoses, pentoses, uronic acids and hexosamines [1–4].

The components of bacterial capsules are also of great heterogeneity, containing pure cellulose, dextrose, hyaluronic acid and many mixed polysaccharides [5, 6], among others. The composition of the cell wall of fungi is also of great variety, being built up of chitin, peptidoglucomannan or cellulose [7–10].

The most common component of the cell walls of algae is pectin, associated with other oligo- and polysaccharides and sulphated polysaccharides [11, 12].

Present knowledge of the carbohydrate components of the cell walls of microorganisms mainly stems from chemical analyses, and information about the ultrastructural order of their carbohydrate components is lacking due to the lack of an appropriate morphological method. In fact, the PAS reaction, a selective histochemical reaction of vicinal OH groups, furnished

evidence of the presence of such groups in bacterial and fungal cell walls. Since this reaction does not induce anisotropy effects on the reactive structures, it gives no information about the structural order of these groups within the cell walls. The same applies to the electron microscopical methods of vicinal glycol groups based on their reduction of silver or alkaline bismuth [13, 14].

Recently, an oriented topo-optical staining reaction (aldehyde-bisulphite-toluidine blue-ABT) of vicinal OH groups has been developed in our institute [15]. Under the polarization microscope the reaction is able to offer information about their linear order in different biological structures [16-18].

In the present paper we shall report on findings on microbial cell walls obtained by the ABT reaction indicating a definite and varying molecular order of their carbohydrate components.

Material and methods

The material for investigation was collected from natural sources or was obtained from agar or blood agar cultures. In some cases the microbes were studied in histological sections of infected tissues. The species investigated are listed in Table I.

Suspensions from the cultures were dropped onto the slide and after drying at room temperature fixed by heat or in 5% glutaraldehyde in 0.15 M phosphate buffer pH 7.0. The tissues were fixed in 10% neutral formaldehyde and embedded in paraffin.

Aldehyde-bisulphite-toluidine blue (ABT) reaction was performed as described earlier [15]. The smears or sections are treated with 0.5-1% periodic acid for 30 min, by which the vicinal OH groups are transformed into dialdehyde groups; then treatment followed for 20-30 min with saturated Na-bisulphite solution, in which the aldehyde groups bind bisulphite and are rendered negatively charged and able to bind cationic dyes at low pH values (Fig. 1a). After staining with toluidine blue at pH 1.0 (0.1% toluidine blue in 0.1 N HCl) for 5 min, and stabilization of the staining with 1% potassium ferricyanide [19], the preparations were covered with gum arabic containing 1% potassium ferricyanide. The dried slices were sealed with Canada balsam.

After ABT treatment, carbohydrate components with 2-3 glycol groups are characterized by intensive metachromatic basophilia [20, 21] and by strong toluidine blue-induced birefringence [15], when the vicinal OH groups are present in linear order within the reacting structures [16-18]. Optical analyses of different well-known linear polysaccharides indicated that the birefringence induced by ABT was negative with respect to the length of the reacting polysaccharide chains. Based on this optical evidence, it became possible to detect the molecular order of the vicinal OH groups and in turn the polysaccharides to which they are attached in biological substrates: a possibility not shared by other methods of ultrastructural research.

Strong basophilia after ABT reaction without birefringence effect, on the other hand, is an indication of the presence of vicinal OH groups in great density but in random distribution, accordingly dye binding is disordered and no anisotropy effects are induced [15].

Trypsin treatment prior to the ABT reaction was carried out to eliminate enzyme sensitive, non-structural proteins which could interfere either with the reaction of the vicinal OH groups or with the orientation of the bound dye molecules. After being covered with 0.3% celloidin, the smears and slices were treated with 2 mg/ml trypsin (Serva) in phosphate buffer (pH 8.2) at 37°C for 2 hr.

For enzymatic degradation of the carbohydrate components, treatment with α -amylase (Merck, 1 mg/ml), with diastase (Serva, 2 mg/ml) in distilled water, pectinase (Serva, 8 mg/ml) in acetate buffer pH 4.5, and with lysozyme (Serva, 1 mg/ml) in pH 4.0 acetate buffer containing 0.1% EDTA were performed at 37°C for 2 hr.

Table I
Birefringence after the ABT reaction

Species studied	Sign of birefringence with respect to cell surface	Retardation
<i>Algae</i>		
<i>Chroococcus</i>	—	50 nm
<i>Oscillatoria</i>	—	45 nm
<i>Anabena</i>	—	55 nm
<i>Fungi</i>		
<i>Psalliota campestris</i>	—	35 nm
<i>Aspergillus</i>	—	25 nm
<i>Penicillium</i>	—	20 nm
<i>Trichophyton rubrum</i>	—	35 nm
<i>Saccharomyces cerevisiae</i>	+	45 nm
<i>Candida albicans</i>	+	50 nm
<i>Cryptococcus neoformans</i>	+	70–100 nm
<i>Actinomycetales</i>		
<i>Actinomyces</i> (from tonsilla)	+	30 nm
<i>Streptomyces griseus</i>	—	35 nm
<i>Cell walls of bacteria</i>		
<i>Bacillus subtilis</i>	+	35 nm
<i>Bacillus anthracis</i>	+	40 nm
<i>Lactobacillus caucasicus</i>	+	35 nm
<i>Escherichia coli</i> A ⁻	+	20 nm
<i>Salmonella minnesota</i> R	+	15 nm
<i>Shigella sonnei</i> R	+	5 nm
<i>Bacterial capsules</i>		
<i>Diplococcus pneumoniae</i>	—	35 nm
<i>Klebsiella</i>	—	40 nm

Results

Staining and optical characteristics of algae. One species of each of the genera *Oscillatoria*, *Anabena* and *Chroococcus* was investigated. The micelial species of *Oscillatoria* and *Anabena* failed to stain with toluidine blue at pH 1.0 (in 0.1 N HCl) and showed a very weak birefringence in water (Plate I, 1a, 1b). They began to take up toluidine blue from pH 3.0 upwards, attributable to the COOH groups of uronic acid components of pectin type polysaccharides. After the ABT reaction the *Oscillatoria* showed intensive metachromatic basophilia with very strong birefringence (45 nm retardation)

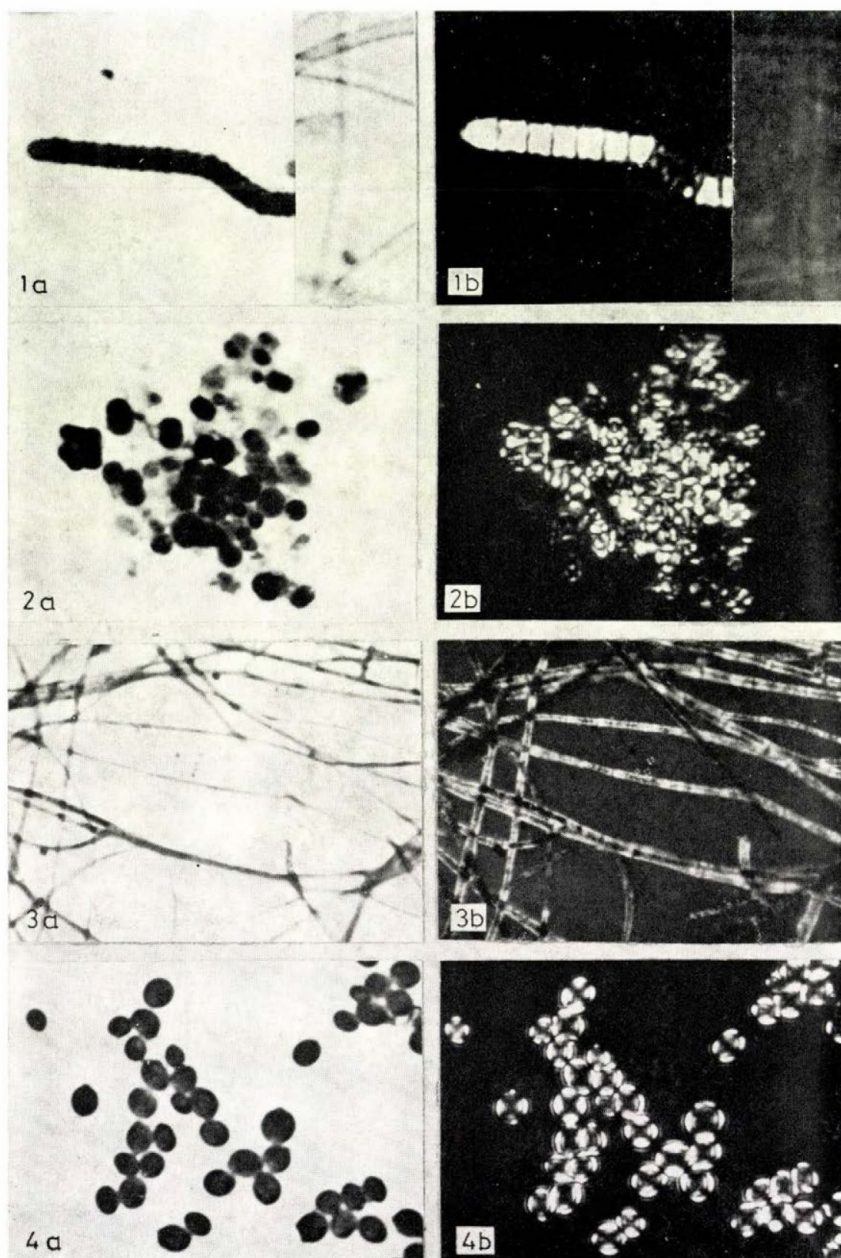


Plate I. 1a and 1b. *Oscillatoria*, blue alga, ABT reaction. (a) Under the light microscope; (b) with crossed polars. In (a) intensive basophilic (metachromatic) staining of cell walls is seen, in (b) the ABT induced strong birefringence, negative with respect to the surface. In controls, *Oscillatoria* failed to stain with toluidine blue pH 1.0 and showed no birefringence; see inserts Plate I. 2a and 2b. *Chroococcus* stained with toluidine blue at pH 1.0. (a) Under the light microscope; (b) with crossed polars. In (a) there is intensive basophilia and in (b) strong birefringence in the form of optically positive spherites. Since *Chroococcus* shows this optical

negative with respect to the surface of the wall, and red polarization colour (Plate I 1a, 1b). On the other hand, *Chroococcus* showed already in control preparations an intensive staining reaction with toluidine blue at pH 1.0, and revealed strong birefringence of the capsules, in the form of optically positive spherites (*i. e.* negative with respect to the surface) (Plate I 2a, 2b). This indicated the presence in their capsules of acid polysaccharides in tangentially oriented pattern. The ABT reaction did not cause a further increase in staining effect nor in the toluidine blue-induced birefringence. The polarization colour was red; it turned into green on staining with 0.01% toluidine blue at pH 1.0.

Staining and optical characteristics of fungi. Unstained molds: *Penicillium*, *Aspergillus*, *Trichophyton rubrum* and the hyphae of the basidiomycetes *Psalliota campestris*, showed a weak birefringence in water, which faded away in gum arabic. Plate I 3a, 3b demonstrate the staining and optical behaviour of *Penicillium* after ABT. The hyphae show intensive (metachromatic) basophilia and strong birefringence of about 20 nm retardation. The birefringence was negative with respect to the length of the hyphae. *Psalliota*, *Trichophyton* and *Aspergillus* revealed similar staining and optical behaviour with ABT.

Yeasts. Unstained *Saccharomyces cerevisiae* and *Candida albicans* displayed no birefringence of their cell walls either in water or in gum arabic. They also remained unstained on control staining with toluidine blue at pH 3.0. In both species, the ABT reaction induced a very strong basophilic staining and birefringence, with about 45–50 nm retardation (in the form of optically negative spherites, *i. e.* positive with respect to the cell surface), in contrast to the molds and *Psalliota campestris* (Plate I 4a, 4b). *Cryptococcus neoformans* in brain failed to stain with toluidine blue at pH 1.0 and showed no birefringence. After toluidine blue staining at pH 3.0, the capsules of cryptococci showed basophilic staining and birefringence in the form of negative spherites, with about 15–25 nm retardation as shown by MOLNÁR [22]. The ABT reaction induced a very intensive basophilia and strong birefringence of the slimy capsules, with about 70–100 nm retardation. Optically they were seen as negative spherites (Plate II 5a, 5b).

Staining and optical characteristics of bacterial cell walls. For comparison, *Actinomycetales*, Gram-positive and Gram-negative, capsulated and

effect without any pretreatment, this staining effect and optical character may be attributed to acid mucopolysaccharides.

Plate I. 3a and 3b. Penicillium, ABT reaction; (a) under the light microscope; (b) with crossed polars. The basophilic hyphae are strongly birefringent (negative with respect to their length), indicating perpendicularly oriented dye molecules, and linearly oriented micellar polysaccharide chains in the cell wall.

Plate I. 4a and 4b. Saccharomyces cerevisiae, ABT. (a) Under the light microscope; (b) with crossed polars. The basophilic cell walls show strong birefringence (in the form of optically negative spherites), indicating surface parallel orientation of the dye molecules and radially oriented vicinal OH groups

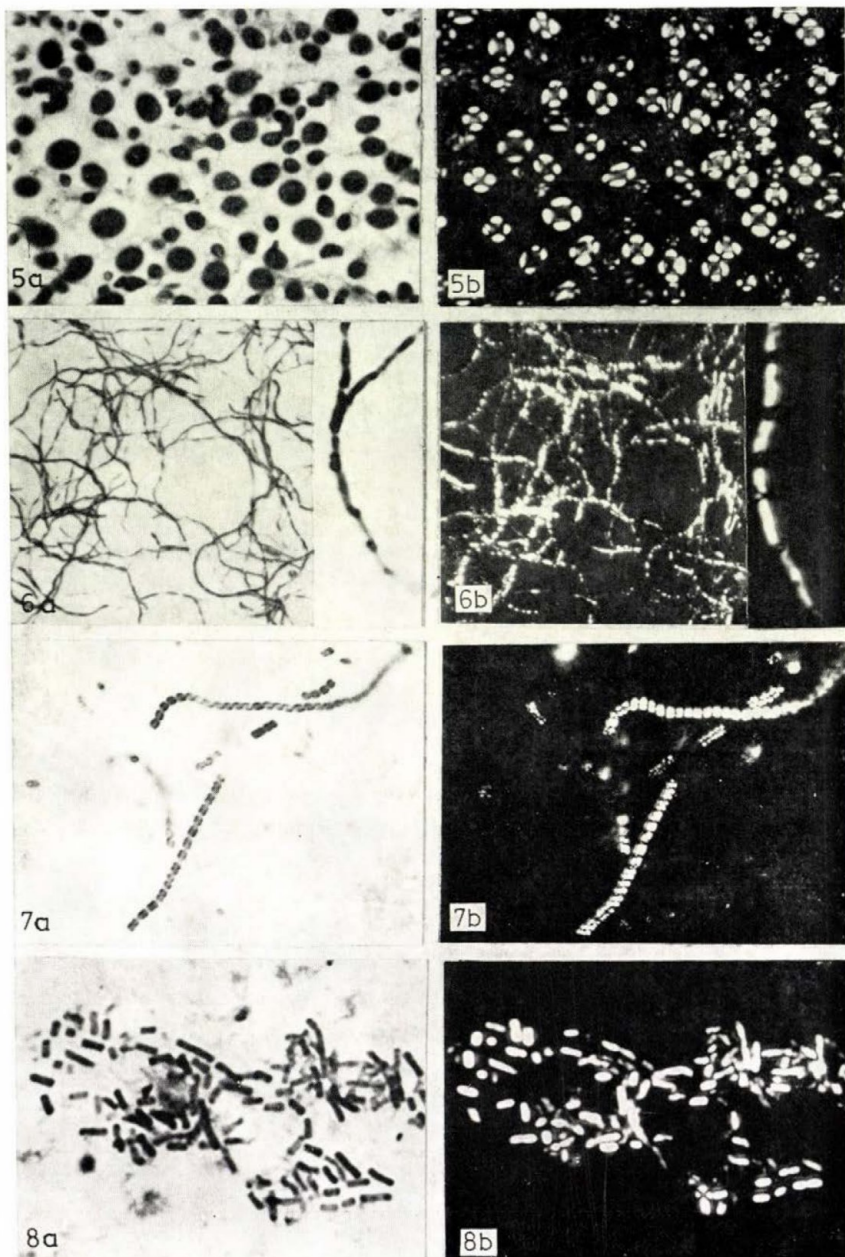


Plate II. 5a and 5b. *Cryptococcus neoformans*, ABT, in tissue section of brain (kindly supplied by Dr. Sz. GOMBA, Institute of Pathology, University Medical School, Debrecen). The strongly basophilic cryptococci (a) show intensive birefringence (b) in the form of negative spherites. Plate II. 6a and 6b. *Streptomyces griseus*, streptomycin-producing, (kindly supplied by Dr. S. VITALIS, Institute of Biology, University Medical School, Debrecen), ABT reaction; (a) under light microscope; (b) with crossed polars. The basophilic hyphae show strong birefringence, negative with respect to their length, indicating a linearly oriented polysaccharide structure in the cell walls of the hyphae. The inserts show mycelia at higher magnification.

noncapsulated bacteria were investigated. In unstained preparations no measurable birefringence was observed.

Actinomycetales. Hyphae of *Actinomyces* species, often seen in tonsillar biopsies, did not stain with toluidine blue at pH 1.0. The ABT reaction induced a strong basophilia and birefringence (30 nm), positive with respect to the length of the hyphae, indicating a tangential orientation of dye molecules to the surface. The streptomycin-producing *Streptomyces griseus* showed an opposite optical behaviour with negative birefringence (about 35 nm), indicating a dye molecule orientation perpendicular to the surface (Plate II 6a, 6b). In controls stained with toluidine blue pH 1.0, *Streptomyces* showed a very weak metachromasia and negative birefringence with about 3–4 nm retardation.

Gram-positive bacteria. The cell walls of Gram-positive bacteria such as *Lactobacillus caucasicus* (Plate II 7a, 7b), *Bacillus subtilis* and *Bacillus anthracis* (Plate II 8a, 8b) revealed strong ABT-induced staining and birefringence. The intensively basophilic cell walls appeared strongly birefringent with about 35–40 nm retardation and green-yellowish in colour. The birefringence was positive with respect to the cell wall surface.

The protein capsules of *B. anthracis*, known to be built up of d-glutaminic acid polymer, remained unstained after the ABT reaction, indicating the absence of carbohydrates in their structure.

Amylase, diastase and trypsin treatment did not alter the intensity of the ABT reaction of bacterial cell walls. Lysozyme treatment on the other hand markedly decreased the staining and birefringence of both *B. anthracis* and *L. caucasicus*.

Gram-negative bacteria. In general after the ABT reaction Gram-negative bacteria showed weaker basophilia and birefringence than Gram-positive bacteria. The birefringence was especially weak, often hardly measurable, on the S forms of bacteria (*Salmonella minnesota* S, *Shigella sonnei* S and *Escherichia coli* A⁺). The R and A⁻ forms of the same species showed intensive birefringence with retardations of about 15–20 nm on *E. coli* A⁻ and *S. minnesota* R (Plate III 9a, 9b, 10a, 10b), and only of 5–6 nm on *S. sonnei* R. The sign of birefringence was positive with respect to the cell surface as seen with the Gram-positive bacteria.

Staining and optical characteristics of polysaccharide capsules of bacteria after ABT reaction. In unstained smears of capsulated *Diplococcus pneumoniae*

Plate II, 7a and 7b. *Lactobacillus caucasicus*, ABT. (a) Under the light microscope; (b) with crossed polars. The cell walls are strongly basophilic and birefringent, positive with respect to the cell surface

Plate II, 8a and 8b. *Bacillus anthracis*, ABT. (a) Under the light microscope; (b) with crossed polars. The basophilic cell walls show strong birefringence (positive with respect to the surface) indicating a radial distribution of the reacting OH groups in the cell wall. The proteinaceous capsules failed to stain

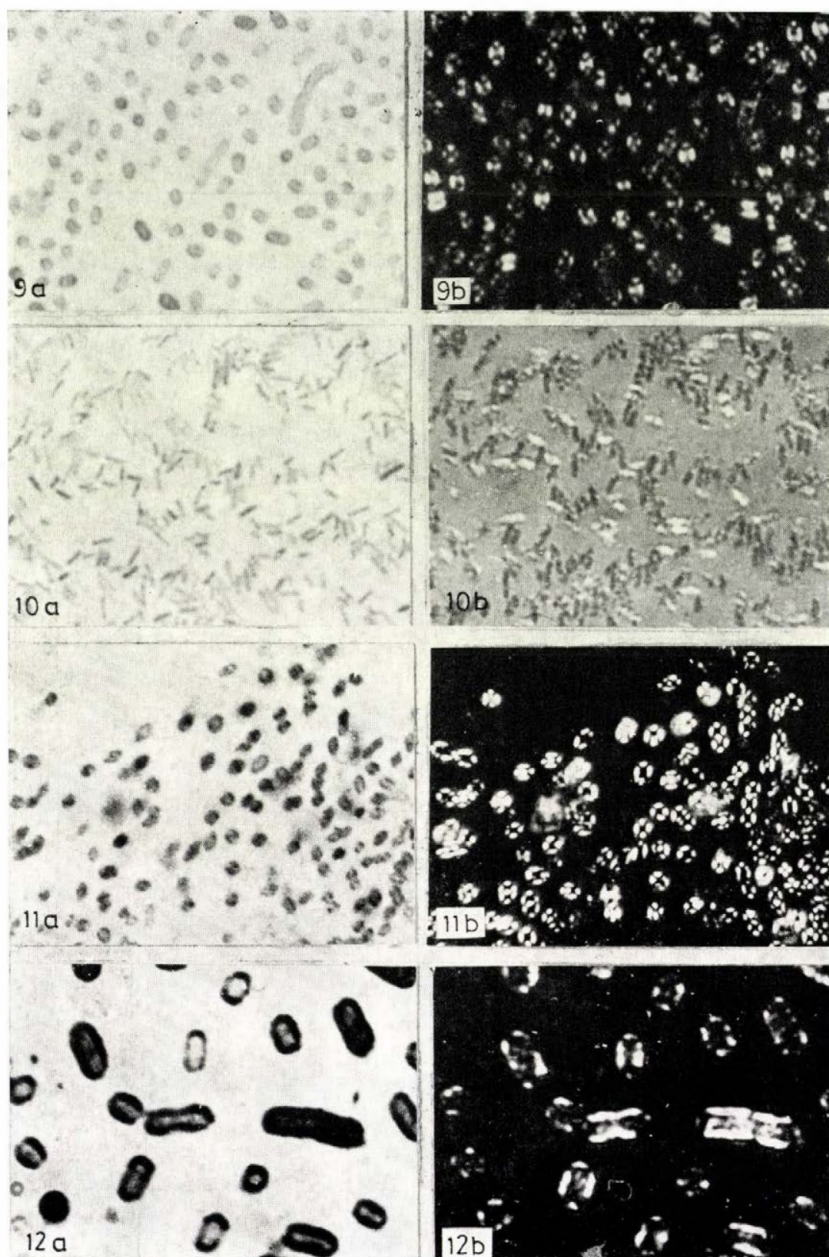


Plate III, 9a and 9b. *Escherichia coli* A⁻, ABT. (a) Under the light microscope; (b) with crossed polars. The individual bacteria appear partly as spherites and partly as rods. The sign of birefringence is positive with respect to the length of the rods

Plate III, 10a and 10b. *Salmonella minnesota*, R mutant, ABT. (a) Under the light microscope; (b) with crossed polars, at additive compensation of 6 μ m. The horizontal bacteria are light, and the vertical ones are dark, indicating positive birefringence

Plate III, 11a and 11b. *Diplococcus pneumoniae*, ABT. (a) Under the light microscope; (b) with crossed polars. The capsular polysaccharides show strong basophilia and birefringence in the

and *Klebsiella*, no birefringence could be observed, and they failed to stain with toluidine blue pH 1.0. At pH 3.5, they showed weak metachromasia and birefringence. After the ABT reaction (Plate III 11a, 11b, 12a, 12b), intensive metachromatic basophilia of the capsules was seen with strong birefringence in the form of optically positive spherites, *i. e.* with a radial orientation of the slow axis of retardation.

Discussion

The ABT reaction of vicinal OH groups has been used to study in the polarization microscope the molecular structural order of carbohydrate components in microbial cell walls and capsules. The reaction that has been developed as an oriented topo-optical staining reaction of linearly ordered vicinal OH groups in biological structures [15], is characterized by intensive, selective basophilic staining with toluidine blue at pH 1.0 of carbohydrate compounds containing vicinal OH groups, and by strong toluidine blue-induced birefringence, when the vicinal OH groups are present in a linear order.

According to our observations, the ABT reaction is eminently suitable for the selective demonstration of carbohydrate components and for the detection of their linear order in microbial cell walls and capsules.

Since there is strong evidence [15] that the birefringence induced by the ABT reaction is negative with respect to the chain length of the reacting linear polysaccharides, the reaction provides information about the molecular structural order of carbohydrates in microbial cell walls and capsules, a possibility not offered by other ultrastructural methods. Fig. 1 serves to enlighten the molecular structural interpretations of the birefringence phenomena induced by ABT. As seen, the sign of birefringence is negative with respect to the chain length of the reacting polysaccharides.

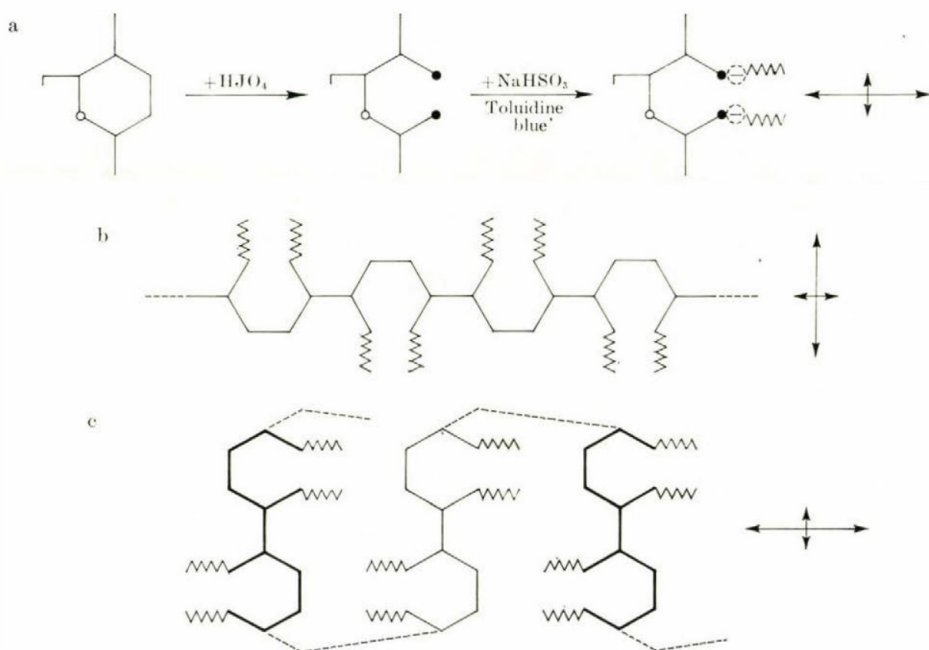
Algae. The blue algae showed after the ABT reaction (pH 1.0) strong basophilia and birefringence. The birefringence was negative with respect

form of optically positive spherites, *i. e.* with radial orientation of the slow axis of retardation, indicating radial dye molecule orientation, and circularly oriented texture of the reacting polysaccharide structure

Plate III, 12a and 12b. *Klebsiella*, ABT, (a) Under the light microscope; (b) with crossed polars.

Very strong basophilia and birefringence, negative with respect to the length of the rods

Fig. 1. Schematic demonstration of the molecular structural mechanism of the birefringence effect of polysaccharide chains induced by ABT. The dialdehydes induced by periodic acid oxidation of vicinal OH groups are rendered negative by addition of bisulphite and able to bind toluidine blue at pH 1.0. (a) Oriented dye binding manifests with strong birefringence, negative with respect to the length of the polysaccharide chains. (b) Negative birefringence with respect to the surface of the cell wall (or optically positive spherites) is indicative of perpendicularly oriented fibrillar polysaccharides. (c) Positive birefringence with respect to the cell surface (optically negative spherites), induced by ABT is indicative of tangentially oriented dye molecules and of radially oriented linear OH groups, resulting probably from radial or helical polysaccharide chains



to the cell surface, indicating longitudinally oriented linear carbohydrate chains in the cell walls. *Chroococcus* species revealed without any pretreatment basophilic metachromatic staining with toluidine blue at pH 1.0 and a strong birefringence suggesting that the slimy capsule of *Chroococcus* is built up of sulphated acid polysaccharide chains which bind toluidine blue at pH 1.0. The finding that the ABT reaction did not further increase the staining effect and birefringence indicated that this acid polysaccharide did not contain free vicinal OH groups for the ABT reaction. (The cell walls of *S. griseus* showed without pretreatment a weak topo-optical staining reaction with toluidine blue at pH 1.0, which was strongly increased by successive ABT reactions, indicating the presence of sulphated and not sulphated carbohydrates, with free vicinal OH groups.)

Fungi. The cell walls of fungi show great differences in chemical composition. While in molds and basidiomycetes the main compound is chitin, in yeasts it is peptidoglucomannan. Our optical findings point to differences in steric orientation of the carbohydrates of cell walls in these microorganisms.

Molds (*Penicillium*, *Aspergillus*, *Trichophyton*) and the basidiomycetes *P. campestre* with chitinous cell walls revealed after ABT a strong negative birefringence — with green polarization colour, suggesting a high degree of dye molecule orientation, perpendicular to the surface, indicating the presence of carbohydrate chains oriented parallel to the cell surface in the chitinous cell wall. These results are in agreement with the finding that cellulose and

chitinous cell walls have the same linearly oriented fibrillary structure [23]. It is known that periodic acid oxidizes also the acylamino-hydroxyl groups of chitin, similarly to the vicinal OH groups to dialdehydes [24, 25] and therefore it reacts with ABT.

In yeasts, in contrast to the cell walls of molds, the sign of birefringence induced by the ABT reaction was positive with respect to the cell surface, suggesting a radial or helical structure of the peptidoglucomannane polysaccharide chains in the cell walls (see Fig. 1c).

Bacterial cell walls. The most prominent component of bacterial cell walls is their mucopeptide framework. The mucopeptides are present in greater amounts in Gram-positive than in Gram-negative bacteria [1]. Accordingly, the birefringence induced by the ABT reaction was always stronger in Gram-positive than in Gram-negative bacteria. Treatment with lysozyme, an enzyme specific for the glycan chains of mucopeptides, resulted in a marked decrease of the cell wall birefringence induced by the ABT reaction. KELEMEN and ROGERS [26] and ROGERS [4] assumed two possibilities for the orientation of mucopeptides in the bacterial cell walls with radial or tangential orientation of the glycan chains. The positive sign of birefringence with respect to the cell surface, as found in both Gram-positive and Gram-negative bacteria, suggested a radial-linear orientation of vicinal OH groups, resulting probably from the strongly spiralized macromolecular glycan chains in the cell wall. The first polysaccharide known to have a helical structure was amylose. Further polarization optical studies on pure unstained xylan crystals [27] and Ca-pectate gels [28] indicate that they also may have a helical structure. The helical conformation of many polysaccharides has been suggested by KIRKWOOD [23].

Polysaccharides of bacterial capsules. The weak staining and optical effect of pneumococcal polysaccharide capsule with toluidine blue (pH 3.0) may be attributed to the COOH groups of the uronic acid components, as described by WOOD and SMITH [29] and by WESTPHAL and LÜDERITZ [6]. The ABT reaction induced an intensive metachromatic basophilia and a strong radially positive birefringence, (negative with respect to the cell wall surface), indicating radially oriented dye molecules and thus tangentially oriented extended polysaccharide chains in the capsule (see Fig. 1b). Similar staining and optical behaviour were shown by the capsular polysaccharides of *Klebsiella*.

The opposite optical character of birefringence of microbial cell walls after ABT reaction should be interpreted as evidence of a different molecular orientation of their carbohydrate components. A negative birefringence with respect to the length of the cell wall surface is indicative of tangentially oriented linear polysaccharide structures (cellulose, chitin cell walls and capsular polysaccharides of bacteria), and a positive birefringence suggests vicin-

al OH groups oriented perpendicularly to the cell wall surface. This may reflect a helical conformation of carbohydrate compounds (yeasts and bacterial cell walls).

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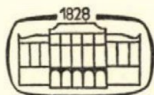
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REGULATION OF METHIONINE SYNTHESIS IN SACCHAROMYCES CEREVISIAE OPERATES THROUGH INDEPENDENT SIGNALS: METHIONYL-tRNA^{met} AND S-ADENOSYLMETHIONINE*

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(Received April 6, 1975)

Summary. The synthesis of at least six enzymes implicated in methionine biosynthesis in *Saccharomyces cerevisiae* is regulated pleiotropically by two independent regulatory systems. Repression of enzyme synthesis is promoted either by exogenous methionine or by exogenous S-adenosylmethionine (SAM). The regulatory system acting in methionine mediated repression seems to comprise methionyl-tRNA^{met} as a co-repressor and the other system, acting in SAM repression, comprises SAM as a co-repressor. This concept gives a role in regulation to the two activated forms of methionine. Moreover, evidence is presented that the "SAM repressor" probably acts at a post-transcriptional level while the "met-tRNA^{met} repressor" would be active at the transcriptional level. These conclusions have been based on two series of experiments: one using a mutant bearing a modified methionyl-tRNA synthetase [L-methionine: tRNA^{met} ligase (AMP-forming) E.C.6.1.1.10] and one studying the kinetics of depression of synthesis of one of the biosynthetic enzymes after repression either by exogenous methionine or by exogenous SAM. Our results are strengthened by the use of two different drugs: one inhibiting messenger RNA synthesis and the other inhibiting protein synthesis.

The pathway of methionine biosynthesis in *Saccharomyces cerevisiae* is shown in Fig. 1. Studies of the metabolic regulations acting on this pathway allowed the following conclusions. 1. The regulatory effect of methionine on its own biosynthetic pathway is achieved through repression of enzyme synthesis as shown by the kinetic study of the overall methionine biosynthesis *in vivo* [1]. The repressive effect of methionine has been evidenced on the synthesis of a number of methionine biosynthetic enzymes such as homoserine O-transacetylase, homocysteine synthetase, ATP sulphurylase, sulphite reductase [2] and sulphate permease [3]. They have been called methionine group I enzymes (met enzymes). 2. The synthesis of the above enzymes appears to be coordinated although no linkage has been found between the structural genes encoding for at least three of them [4–6]. Mapping of the other genes has not yet been done. 3. The pleiotropic regulatory system acting on the synthesis of the met enzymes involves different proteins coded by genes ETH2, ETH3, ETH10 [7, 8] and one of the activated products of methionine, methionyl-tRNA^{met} (met-tRNA^{met}) [2, 9]. 4. Another regulatory effector

* The substance of this paper was presented in a Colloquium on Regulation of Amino Acid Biosynthesis in Microorganisms, Ninth Meeting of the Federation of European Biochemical Societies, Budapest, August 25–30, 1974.

** HUGUETTE DE ROBICHON-SZULMAJSTER died prematurely in April, 1974. She had initiated this work and we hope that this paper will honour her memory.

detected recently is S-adenosylmethionine (SAM), the other activated product of methionine. Exogenous SAM leads to repression of synthesis of the same enzymes as methionine [10]. This regulatory effect of SAM is not due to some reconversion into methionine [10].

At this point of the work, two hypotheses arose. One is that the regulatory system acting on methionine biosynthesis involves only one co-repressor, the met-tRNA^{met}; in this case, it could be assumed that SAM as the only methyl donor of the cell, exerts its regulatory function through an indirect effect due to some modification in the methylation of specific nucleosides important for the met-tRNA^{met} to perform its regulatory function. The second hypothesis is that the regulation of methionine biosynthesis in *S. cerevisiae* comprises two different systems, one involving met-tRNA^{met} and the other involving SAM, as co-repressors.

Our recent results are in favour of the second hypothesis. We have two kinds of evidence, one derived from the study of a mutant bearing a genetically modified methionyl-tRNA synthetase and one obtained at the study of the kinetics of derepression of the synthesis of one met-enzyme.

Materials and methods

Strains. The haploid strains used for this study were strain 4094-B (α , ural, ade2) from Dr. F. SHERMAN's collection, and strain CC196-6D (α , ural) from Dr. H. CHEREST's collection.

Growth conditions. YNB synthetic medium was used. This medium contains for 1 litre of distilled water, Difco Yeast Nitrogen Base without amino acids, 7 g; D-glucose, 20 g. This medium was supplemented with adenine and uracil, 20 mg per litre. When DL-methionine or S-adenosylmethionine was added, the concentrations are given in the text, tables or figures.

For derepression studies, 1 litre of YNB medium containing DL-methionine or S-adenosylmethionine was inoculated with about 2×10^8 cells per litre. The culture was agitated at 28°C overnight and in the morning the concentration of cells was about 1×10^{10} cells per litre (log phase growth). They were quickly harvested by centrifugation, washed with YNB medium at 28°C and resuspended in prewarmed medium containing adenine and uracil but no methionine or SAM ($t = 0$). The whole process took about 10 min. The culture was agitated at 28°C and at different times 25 ml samples were taken, poured on crushed ice, centrifuged and washed once with phosphate buffer pH 7.5, 100 mM. Homocysteine synthetase was then assayed on the benzene treated cells.

Enzymatic assays. Extraction and assay of homocysteine synthetase and ATP sulphurylase were performed as described previously [10]. For derepression studies, homocysteine synthetase activity was measured on benzene permeabilized cells [7, 18]. The activities were given in nanomoles of substrate transformed $\times$ mg protein⁻¹ $\times$ min⁻¹ or in nanomoles $\times$ min⁻¹ $\times$ mg dry weight⁻¹.

Dry weight. The average of at least 10 determinations made in this laboratory by Dr. J. ANTONIEWSKI was 180 mg dry weight per litre in a culture at $OD_{650}^{1\text{cm}} = 1.0$ measured in a Zeiss PMQII spectrophotometer.

Methionine and SAM pools. The pools were estimated as described previously [10] and expressed in μ moles per gram of dry weight.

Determination of the intracellular amount of tRNA^{met} charged in vivo. Extraction of tRNA^{met} and determination of the per cent of tRNA^{met} charged in vivo were performed as described in [12].

Chemicals. S-adenosylmethionine and cycloheximide were from Sigma Chemical Company. Lomofungin was a generous gift from Dr. G. B. WHITEFIELD of the Upjohn Company, and O-acetyl-DL-homoserine was a generous gift from Dr. M. CHEREST.

Results

1. Study of a mutant bearing a genetically modified methionyl-tRNA synthetase (MTS)

In a previous study [2] the use of a thermosensitive mutant bearing a modified MTS allowed to show that the co-repressor of the pleiotropic regulatory system was tRNA^{met} rather than free methionine [2]. It was reported that the thermosensitivity and the presence of a defective MTS were due to the same genetic lesion [11]. However, during the study of this mutant, we separated these two characters and we selected a strain bearing only the mutation leading to the MTS modification [12]. The latter strain was used for the study presented below.

Table I

Kinetic parameters of methionyl-tRNA synthetase extracted from the reference strain and the MTS deficient strain

Strain*	MTS specific activity**	Km		
		L-met*** (mM)	ATP (mM)	tRNA (mM)
4094-B	1.4	0.005	0.5	0.11
CC196-6D	0.28	0.5	—	—

* 4094-B (α , ade2, ural): reference strain; CC196-6D (α , ural): MTS deficient strain

** Specific activity is expressed in nanomoles L-met charged by mg protein in 10 min. It was measured at 0.1 mM L-met which is saturating for the reference strain

*** L-met: L-methionine

Characterization of the strain. The results reported in Table I show that CC196-6D is a K_m type-mutant. The K_m for methionine of the MTS extracted from the mutant is 100 times higher than the K_m of the MTS extracted from the reference strain. Determination of the K_m values for the two other substrates (ATP and tRNA) is difficult in the case of the mutant enzyme, due to its low affinity to methionine. Nevertheless they seem to be of the same order of magnitude than the values found for the wild type enzyme. It can thus be concluded that the genetic lesion in the mutant CC196-6D results in a much lower affinity of the MTS for methionine.

Repressibility of methionine biosynthetic enzymes and tRNA^{met} charging in vivo. Table II shows that, at 0.2 mM exogenous DL-methionine, the synthesis of two met-enzymes (homocysteine synthetase and ATP sulphurylase) is already repressed to about 50% in the reference strain 4094-B while no repression is observed in the mutant strain CC196-6D. After growth in the presence of 4 mM DL-methionine, synthesis of the two met-enzymes is, however, repressed

to the maximum in both strains. It appears in addition that growth of the mutant strain CC196-6D under non-repressive conditions (minimal medium or 0.2 mM DL-methionine) leads to a low (about 20%) percentage of tRNA^{met} *in vivo*. On the contrary, after growth under repressive conditions (4 mM DL-methionine), the percentage of tRNA^{met} charged *in vivo* is maximum (94%).

Table II

*Specific activity of two biosynthetic enzymes after growth under different conditions
Concomitant percentage of tRNA^{met} charged *in vivo**

Strain*	Addition to minimal medium	Homocysteine synthetase activity**	ATP sulphurylase activity**	Charged tRNA ^{met} <i>in vivo</i> *** (per cent)
4094-B	—	170	106	95
	DL-met 0.2 mM	90	45	90
	DL-met 4 mM	30	13	90
	SAM 0.15 mM	34	6	95
CC196-6D	—	196	89	28
	DL-met 0.2 mM	206	70	20
	DL-met 4 mM	51	9	94
	SAM 0.15 mM	65	11	24

* 4094-B: reference strain; CC196-6D: MTS deficient strain

** Expressed in nanomoles of substrate transformed $\times$ mg protein⁻¹ $\times$ min⁻¹

*** The percentage of tRNA charged *in vivo* was calculated as described in Materials and methods

These results need two comments. First, the correlation between the concentration of methionine in the medium and the amount of met-RNA^{met} present *in vivo* is compatible with the fact that CC196-6D is a *Km* mutant; the tRNA^{met} is probably maximally acylated when the methionine concentration is sufficient to saturate the enzyme. Second, the fact that the per cent of repression of enzyme synthesis parallels the per cent of tRNA^{met} charged *in vivo* is in favour of the role of methionyl-tRNA^{met} as a regulatory effector. However, in the reference strain, 4094-B, the amount of tRNA^{met} charged *in vivo* is at the maximum after growth in all conditions. This has been interpreted to indicate that a minor species of met-tRNA^{met} is more likely to be implicated in the regulatory system than the global tRNA^{met} [2].

After growth in the presence of SAM, synthesis of the met-enzymes is repressed in both strains. But in strain CC196-6D the per cent of tRNA^{met} charged *in vivo* remains low. This indicates that repression by exogenous SAM does not involve the charging of tRNA^{met}.

This is then the first evidence that exogenous SAM and methionine are acting independently, as tRNA^{met} charging parallels methionine mediated repression while SAM can repress enzyme synthesis without any reacylation of tRNA^{met}.

Methionine and SAM pools after growth under different conditions. As we have pointed out in the introduction, the repressive effect of SAM cannot be due to its transformation into methionine since, as it can be seen in Table III, growth in the presence of 0.15 mM SAM does not lead to an increase of the methionine pool in both strains. This has already been shown for the reference strain [10].

Table III

Methionine and SAM endogenous pools in the reference and MTS deficient strain

Strain*	Addition to minimal medium	Methionine pool**	SAM pool**
4094-B	—	1.5	1.1
	DL-met 2 mM	55	12
	SAM 0.15 mM	2.4	23
CC196-6D	—	1	1.3
	DL-met 4 mM	86	3.7
	SAM 0.15 mM	0.88	9.5

* 4094B: reference strain; CC196-6D: MTS deficient strain

** Expressed as manomoles per mg of dry weight

If we consider now the pool size of methionine and SAM after growth in the presence of methionine at high concentrations, it can be seen in Table III that both pools are high in the reference strain 4094-B. Thus, the repression of enzyme synthesis observed in the presence of exogenous methionine could be due to its transformation into SAM. If this hypothesis is correct, the implication of the met-tRNA^{met} in the regulation would not be necessary and the modification in the percent of tRNA^{met} charged *in vivo* observed in the mutant strain CC196-6D would be considered a phenomenon independent of regulation. If, however, we consider the mutant strain CC196-6D, it appears that growth in the presence of methionine at the repressive concentration 4 mM leads to a threefold increase in the SAM pool but this does not suffice for promoting a maximum repression of met-enzyme synthesis [10]. For the moment we have no explanation for the fact that high concentrations of exogenous methionine do not lead to a high endogenous SAM pool in the mutant strain. We have only verified that it is not the result of an impaired methionine adenosyl transferase activity (step 9 in Fig. 1). In any case, the absence of a suf-

ficient SAM accumulation in the mutant strain leads to the conclusion that the repressive effect of exogenous methionine does not require the intermediate formation of SAM.

In conclusion, we think that the above study of repression of enzyme synthesis, tRNA^{met} charging and endogenous pools of the MTS modified mutant (CC196-6D) is a good evidence that exogenous methionine and SAM act independently in the regulation of methionine synthesis in *S. cerevisiae*.

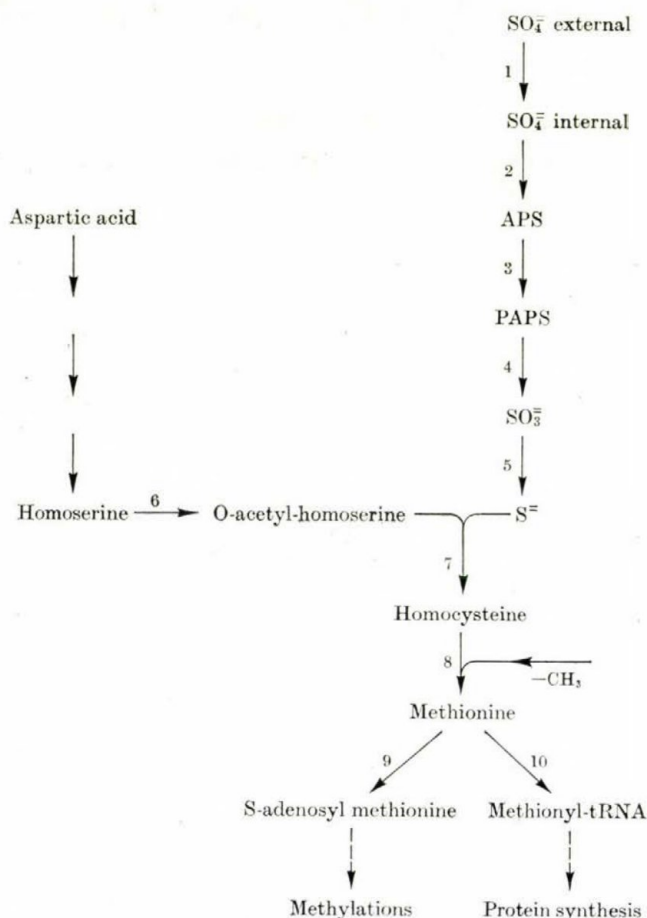


Fig. 1. Methionine biosynthesis in *S. cerevisiae*. APS = adenosyl-5'-phosphosulphate; PAPS = 3'-phospho-adenosyl-5'-phosphosulphate. Enzymes: sulphate permease: step 1; ATP sulphurylase (ATP: sulphate adenylyl transferase, E.C.2.7.7.4): step 2; Sulphite reductase (hydrogen sulphide nicotinamide adenine dinucleotide phosphate oxidoreductase, E.C.1.8.1.2): step 5; Homoserine-O-transacetylase (acetyl CoA: L-homoserine-O-acetyl transferase, E.C.2.3.1.31): step 6; Homocysteine synthetase (O-acetyl L-homoserine acetate lyase, E.C.4.1.99.10): step 7; Methionine adenosyl transferase (adenosine triphosphate (ATP): L-methionine S-adenosyl transferase, E.C.2.5.1.6): step 9; Methionyl-tRNA synthetase (L-methionine: soluble RNA ligase (adenosine monophosphate) E.C.6.1.1.10): step 10

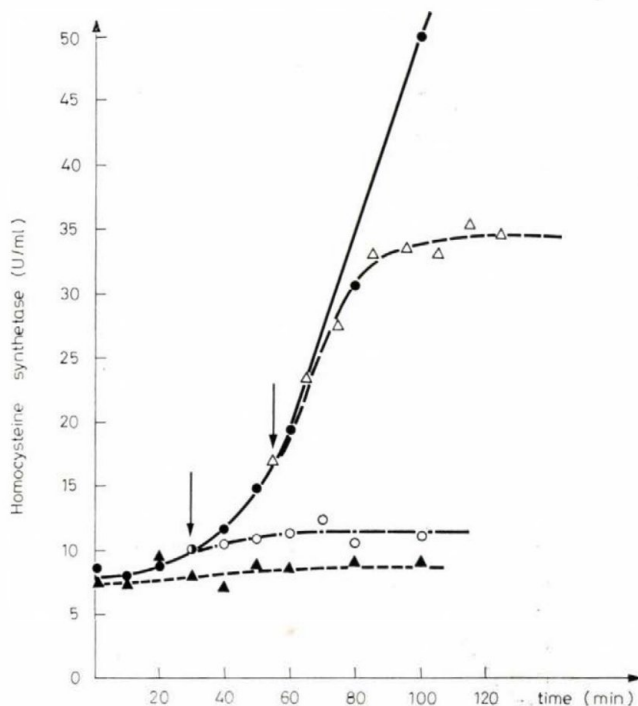


Fig. 2. Release of methionine mediated repression of HCS synthesis. Action of lomofungin added at different times. HCS synthesis was repressed by exogenous DL-methionine, 0.6 mM. Release of repression was done as described in Materials and methods. HCS activity: ●—● without any addition (release of repression); → addition of lomofungin at 10 μ g/ml; ▲—▲ at $t = 0$ min; ○—○ at $t = 30$ min; △—△ at $t = 55$ min

2. Study of the kinetics of derepression of one of the methionine group I enzymes

In the hope to gain some insight at the level of action of the two regulatory systems, we studied the kinetics of derepression of one of the met-enzymes after repression by exogenous methionine or exogenous SAM. Moreover, we studied the action on this derepression of the two antibiotics: cycloheximide and lomofungin. Cycloheximide is an antibiotic known to inhibit protein synthesis [13] and lomofungin has been shown to inhibit messenger RNA synthesis in yeasts [14–17].

As we had already shown that the synthesis of met-enzymes is coordinated, we chose homocysteine synthetase (HCS) as a type enzyme, for the technical reasons emphasized in Materials and methods.

Derepression of homocysteine synthetase (HCS) synthesis after repression by exogenous methionine: action of lomofungin. Fig. 2 shows that after repression by exogenous methionine and elimination of methionine from the medium, the derepression of HCS synthesis begins after a lag of 30 to 40 min during

which HCS is synthesized at the repressed level. After this lag, there is an acceleration in the rate of synthesis during 30 min and then the maximum rate of synthesis is attained. If lomofungin is added during the lag period, no HCS is synthesized. If, however, lomofungin is added when the maximum rate of synthesis has been attained, synthesis of HCS takes place during about 20

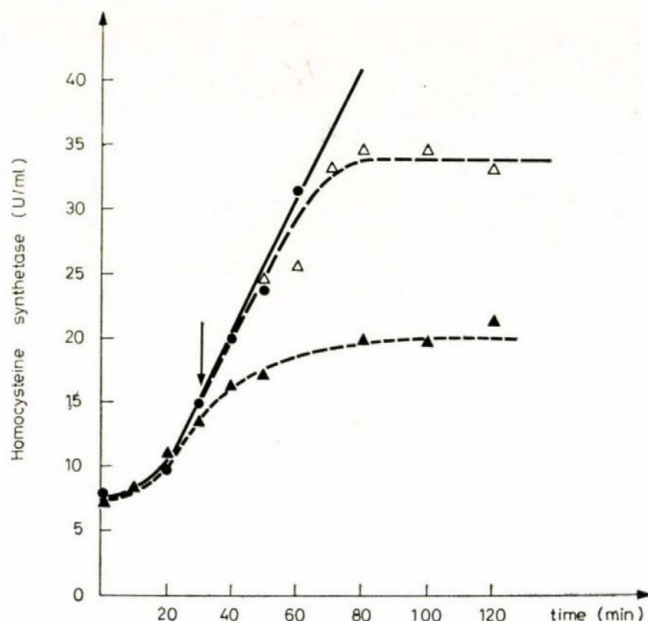


Fig. 3. Release of SAM mediated repression of HCS synthesis. Action of lomofungin added at different times. HCS synthesis was repressed by 0.1 mM exogenous SAM. Release of repression was done as described in Materials and methods. HCS activity: ●---● without any addition (release of repression); → addition of lomofungin at 10 µg/ml; ▲---▲ at $t = 0$ min; △---△ at $t = 30$ min

min. It seems, therefore, that after repression by exogenous methionine, no HCS specific messenger RNA (mRNA) is present in the cell, as the addition of lomofungin preventing any further mRNA synthesis does not allow HCS synthesis. When the maximum rate of synthesis is attained, the addition of lomofungin allows the translation of mRNA present at the moment of addition and the kinetics of HCS synthesis thus reflects the half-like of the HCS-specific mRNA.

Derepression of HCS synthesis after repression by exogenous SAM: the action of lomofungin. In Fig. 3 it is seen that after repression by exogenous SAM, the derepression of HCS synthesis begins immediately after the elimination of SAM from the medium. The rate of synthesis is accelerated during 30 min and then the maximum rate of synthesis is attained. It can be noted that this maximum rate is the same as in Fig. 2.

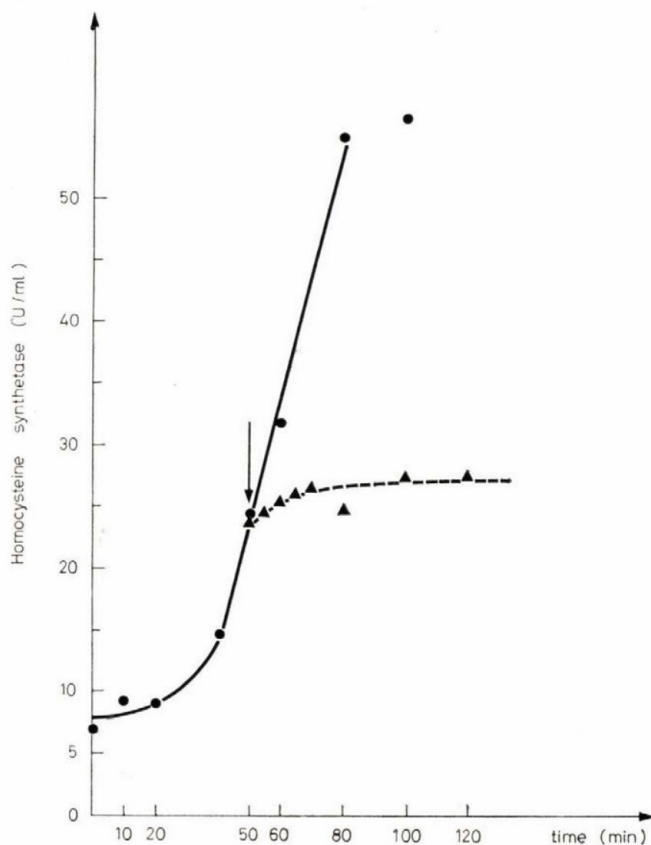


Fig. 4. Effect of cycloheximide on HCS synthesis, after the maximum rate of synthesis has been attained. HCS synthesis was repressed by 0.6 mM exogenous DL-methionine. Release of repression was as described in Materials and methods. HCS activity: ●—● without any addition; ▲----▲ after addition of cycloheximide at 5 μ g/ml

If lomofungin is added at $t = 0$ or $t = 30$ min (maximum rate), synthesis of HCS takes place, showing that even at $t = 0$, HCS specific mRNA was present in the cells.

Effect of cycloheximide added during the derepression of HCS synthesis. For comparison with the effect of lomofungin, we added cycloheximide during the period of maximum rate of synthesis. In Fig. 4 it is seen that HCS synthesis stopped almost immediately as it could be predicted.

Kinetics of repression by methionine or SAM added during derepression of enzyme synthesis. The synthesis of HCS was first repressed by exogenous methionine and derepression was allowed to take place after elimination of methionine from the medium. After the maximum rate of synthesis has been attained, methionine (Fig. 5, A) or SAM (Fig. 5, B) is added to the medium. When methionine is added, the synthesis of HCS is not stopped sharply but

its rate decreases gradually to the repressed rate. In this case, the experimental curve is well above the theoretical curve calculated assuming that when methionine is added, enzyme synthesis is immediately decreased to the repressed level of synthesis.

When SAM is added (Fig. 5, B) the synthesis of HCS ceases almost immediately. The experimental curve in this case looks like the one obtained

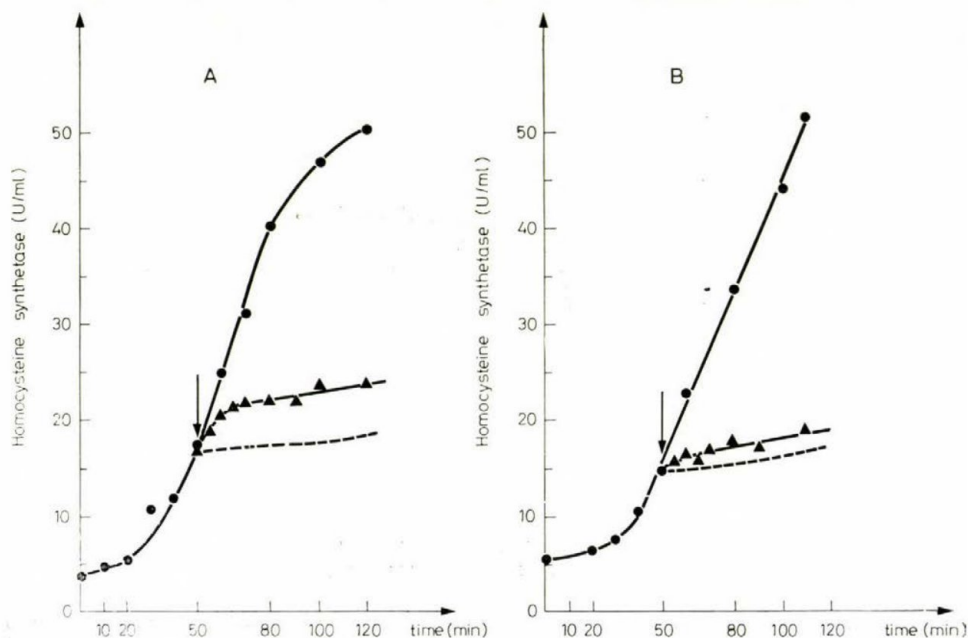


Fig. 5. Effect of methionine or SAM during the release of repression of HCS synthesis. HCS synthesis was repressed by 0.6 mM exogenous DL-methionine. Release of repression was as described in Materials and methods. A: HCS activity ●—● without any addition; ▲—▲ after addition at $t = 50$ min (→) of 0.6 mM DL-methionine. B: HCS activity ●—● without any addition; ▲—▲ after addition at $t = 50$ min (→) of 0.1 mM SAM. In A and B, --- represents the theoretical curve calculated assuming that the preexisting enzyme is diluted and that the repressed level of synthesis begins right at the time of addition of the repressor

after the addition of cycloheximide (Fig. 4) and is a little above the theoretical curve.

We have verified that the difference observed after the addition of methionine or SAM is not due to the activities of the specific permeases for these two metabolites.

Conclusion

The results presented are in favour of the existence in *S. cerevisiae* of two different regulatory systems acting on the biosynthesis of methionine. One system involves S-adenosylmethionine, as an external signal, the other, methionine. This fact is clear when the results obtained with the MTS modified mutant are examined. In this mutant the addition of methionine at high concentration in the growth medium results in (i) a high endogenous methionine pool; (ii) a high concentration of methionyl-tRNA; (iii) a repression of met-enzyme synthesis, but (iv) a low endogenous SAM pool. Addition of SAM to the growth medium results in (i) a high SAM endogenous pool; (ii) a repression of met-enzyme synthesis; but (iii) a low endogenous methionine pool and a low per cent of acylated tRNA *in vivo*.

The presence of a post-transcriptional control exerted by the intermediate of SAM as an exogenous signal is supported by two experiments. 1. The addition at $t = 0$ of lomofungin which stops mRNA synthesis in a few minutes does allow homocysteine synthetase formation; this points to the presence in the cells at $t = 0$ of translatable specific mRNA for homocysteine synthetase. 2. The addition of SAM to a culture actively synthesizing homocysteine synthetase results in a rapid inhibition of this synthesis.

A transcriptional control exerted by the repressor induced by exogenous methionine is supported by two types of result. 1. The addition at $t = 0$ of lomofungin does not allow enzyme synthesis. This points to the absence during methionine mediated repression of translatable specific mRNA for homocysteine synthetase. 2. The addition of methionine to a culture actively synthesizing homocysteine synthetase results in a delayed inhibition of enzyme synthesis.

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REGULATION OF LYSINE BIOSYNTHESIS IN ESCHERICHIA COLI K12*

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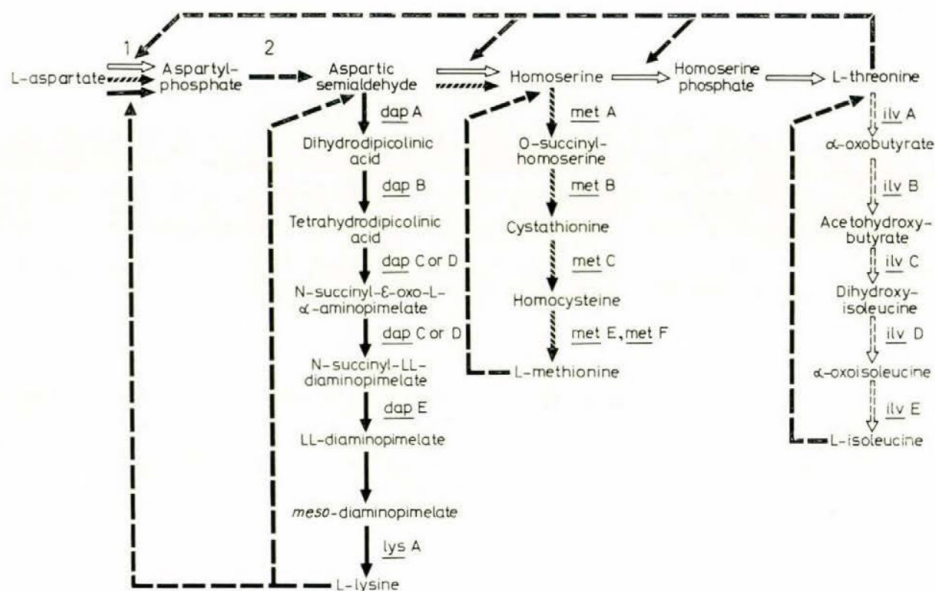
Summary. A general survey of the regulation in lysine biosynthesis in *Escherichia coli* K12 is presented. No polygenic operon exists for the genes that code for enzymes of the lysine biosynthetic pathway. Lysyl-tRNA is not directly involved as a co-repressor in the pathway. Different regulation mechanisms must exist for the different enzymes. In the case of the last enzyme, diaminopimelate decarboxylase, its synthesis is induced *in vivo* by the lysine-sensitive aspartokinase under its non-inhibited allosteric conformation.

In *Escherichia coli*, the diaminopimelate and lysine biosynthetic pathway (Fig. 1) is one of the three pathways which derive from aspartate, that also leads to methionine, threonine and isoleucine. It is now well known [1, 2] that the two first reactions constitute the common part of this branched pathway; the first one, the phosphorylation of aspartate, is catalyzed by three isofunctional aspartokinases, each submitted to specific end-product regulations.

Lysine biosynthesis is performed by nine successive reactions. Lysine-decarboxylase, an inducible enzyme that degrades lysine into cadaverine, and lysyl-tRNA synthetase that catalyzes the specific charge of tRNA_{lys} subsequently used in protein synthesis, may also be considered to belong to the pathway.

Except for the racemase (isomerization of LL-DAP into *meso*-DAP), all the corresponding genes have been mapped on the *E. coli* chromosome [3, 4]. It can be seen (Fig. 2) that all the genes are scattered on the genetic map. No polygenic operon exists in this lysine "regulon". This situation differs considerably from the one observed in the two biosynthetic pathways for which the pattern of regulation is well known, and where the corresponding genes are organized into operons. These are the tryptophan pathway, for which the role of a repressor molecule in JACOB and MONOD's meaning [5] is clearly identified [6, 7]; and the histidine pathway, where no such molecule has been isolated. A role of histidyl-tRNA as a co-repressor has been demonstrated [8]

* The substance of this paper was presented in a Colloquium on Regulation of Amino Acid Biosynthesis in Microorganisms, Ninth Meeting of the Federation of European Biochemical Societies, Budapest, August 25-30, 1974.



Reaction	Name of the enzyme	Abbreviation	Name of the corresponding genes	Regulation of biosynthesis by	Regulation of activity by	References
1	Aspartokinase I	AK I	<i>thr A</i>	thr and ile	thr	(23) (10)
1	Aspartokinase II	AK II	<i>met L</i>	met	0	(1)
1	Aspartokinase III	AK III	<i>lys C</i>	lys	lys	(10)
2	Aspartic semialdehyde dehydrogenase	ASADH	<i>asd</i>	lys, thr, met	0	(15)

Fig. 1. Biosynthetic pathway for the amino acids deriving from aspartate. Symbols: $\longrightarrow$ enzymes repressed by lysine; $\Longrightarrow$ enzymes repressed by threonine plus isoleucine; $\dashrightarrow$ enzymes repressed by methionine; $\equiv\equiv\equiv$ enzymes repressed by isoleucine, leucine and valine. The dashed lines point to reaction subject to feedback inhibition. The symbols in italics represent the genes corresponding to each reaction when this correspondence is known. The symbols for the genes corresponding to reactions 1 and 2 are detailed below

and clear evidence has been offered that the first enzyme of the pathway regulates the expression of the operon at the transcriptional level [9].

After a brief survey of the overall feature of the regulation of the lysine pathway, we shall describe recent experiments indicating that lysyl-tRNA is not directly involved in the regulation as a co-repressor, and that the first enzyme, lysine-sensitive aspartokinase, must play a regulatory role in the synthesis of at least one enzyme of the pathway, i.e. of diaminopimelate decarboxylase.

The gross features of the regulation of the pathway are as follows.

A. Two enzymes are submitted to feedback inhibition of activity by lysine, viz. lysine-sensitive aspartokinase (or AK III) [10], and dihydropyridine synthetase (or DHDP-synthetase) the first enzyme of the linear part of the pathway [11, 12].

B. When the lysine pool in the cell is low, for example in growing lysine auxotrophs in the chemostat, synthesis of most of the lysine enzymes is derepressed: AK III [10], aspartic semialdehyde dehydrogenase (ASA-dehydrogenase), dihydrodipicolinate reductase, deacylase [13], diaminopimelate decarboxylase [14], lysyl-tRNA synthetase (REINISCH and BOY, unpublished). These derepressions are, however, not coordinate. The synthesis of DHDP-synthetase appears constitutive. As to the other enzymes, as their assays are not accurate enough, their regulation is not known.

C. No isofunctional enzymes exist for catalyzing the synthesis of aspartic semialdehyde. In fact, the unique ASA-dehydrogenase is submitted to a multi-valent repression mechanism [15] by lysine *plus* threonine *plus* methionine; but the extent of derepression strongly differs according to the limiting amino acid, lysine limitation being much more effective.

D. The most important role in the regulation of lysine biosynthesis appears to be fulfilled by the feedback inhibition of AK III: most of the mutants selected as lysine analogue resistant or lysine-overproducers were identified as partially or totally feedback resistant mutants of this enzyme activity, the mutation of which maps in the *lysC* gene in which are also mapped the mutations of AK III activity [4, 15].

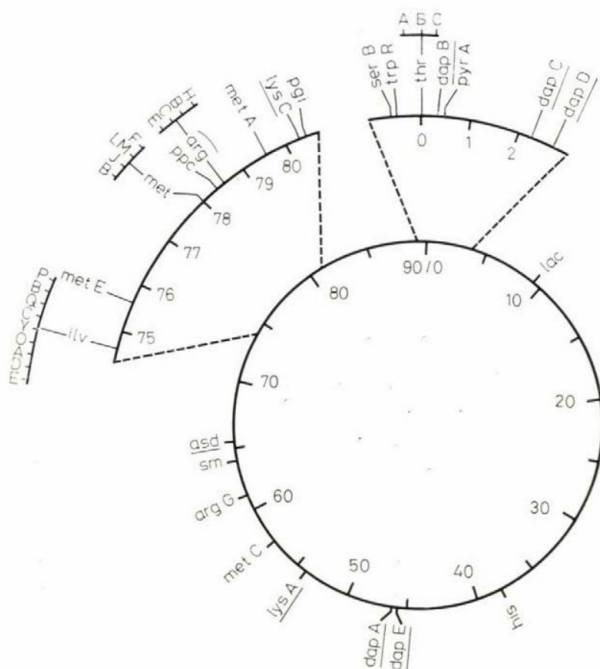


Fig. 2. Location of the structural genes involved in the biosynthesis of diaminopimelate, lysine threonine, methionine and isoleucine. See TAYLOR and TROTTER [3]

The structure and the kinetic properties of this enzyme have been studied in our laboratory; it appears as a good example of the V class of allosteric enzymes [16, 17]. One peculiar feature is the inhibition of the enzyme activity by "non-specific" amino acids such as leucine, isoleucine, phenylalanine, synergistic with the lysine inhibition [18], a synergy well explained by a transconformational allosteric mechanism (MAZAT and PATTE, in preparation). Such synergistic inhibition can play a regulatory role in the cell [19] and fall under the class of "metabolism interlock" as defined by JENSEN [20].

Table I

Kinetic parameters of lysyl-tRNA synthetase of strain L-134

Strains	K_m Lysine ($M \times 10^{-3}$)	K_m ATP ($M \times 10^{-4}$)	K_m tRNA (mg/ml)*
Hfr H	2	1.7	3.5
L-134	70	3.4	3.3

* Expressed as mg of total tRNA per ml. Determination of K_m was performed in cell-free crude extracts by the aminoacylation reaction of tRNA_{lys} (see ref. 8)

E. We have recently isolated from wild-type Hfr H a mutant strain that grows slowly in minimal medium, its growth becoming normal in the presence of 10^{-3} M L-lysine. In this strain, the K_m for lysine of the lysyl-tRNA synthetase appeared to be about 40-fold the wild-type value, the other parameters being of the same order of magnitude than with wild-type strain (Table I).

The percentage of lysyl-tRNA charged *in vivo* has been determined in crude extracts prepared with strain L-134 grown in minimal medium. The obtained value of 7% is extremely low compared to the 64% for wild-type one, as it is usually the case for aminoacyl tRNA synthetase mutants with modified K_m for the amino acid [21].

The specific activity of three enzymes of the pathway was determined with the mutant and wild-type strains being grown in minimal medium or in the presence of lysine in excess. As shown in Table II, the values obtained with strain L-134 when grown in minimal medium were somewhat lower than in the case of the parental strain in the same conditions; on the other hand, the presence of lysine with mutant strain led also to a repression of the synthesis of the enzymes under study, though perhaps not to such an extent as with the wild-type strain.

Thus, though the lysyl-tRNA pool in strain L-134 is extremely low, there is no concomitant derepression of the synthesis of the lysine enzymes we have tested (in minimal medium). We can conclude that, contrary to what

has been observed in the histidine pathway [8], lysyl-tRNA does not play a direct role of co-repressor in the regulation mechanism.

E. Numerous attempts have been made to select regulatory mutants for the synthesis of lysine enzymes. Till recently all have been unsuccessful. To overcome the difficulties due to the multiplicity of the aspartokinases and to the inhibitory role of lysine on AK III activity, we have prepared a strain

Table II

Percentage of tRNA_{lys} charged *in vivo* and total tRNA content in strain L-134

Strains		Doubling time (min)	tRNA charged <i>in vivo</i> per cent		Total content relative to A ₂₈₀ of extract (pmole/OD unit)	
			tRNA _{lys}	tRNA _{met}		
Hfr H	1	46	63	86	24	13
	2	49	65	91	21	11
L-134	1	102	6	84	22	11
	2	110	7.5	88	—	—

Strains were grown in minimal medium. The determination of tRNA charged *in vivo* was performed according to LEWIS and AMES [21]. The results of two independent determinations are given

in which aspartokinases I and II had been lost in the course of successive mutations, the remaining aspartokinase III being moreover desensitized towards lysine inhibition. When we introduced in such a strain a mutation in the ASA-dehydrogenase gene that renders this enzyme activity "leaky", growth of the latter strain was inhibited by the presence in the growth medium of high concentrations of L-lysine. Resistant to this lysine inhibition of growth have then been selected. Among them we have identified an O^c mutant for AK III synthesis. No strain was isolated in which a pleiotropic constitutive synthesis of several lysine enzymes was observed, as it would be the case for a mutation in a common repressor molecule.

G. We have recently obtained evidence of the AK III molecule itself playing a role in the regulation of DAP-decarboxylase synthesis [22]. We have constructed four isogenic strains that differed only in the combination of two mutations: one is a mutation in the *lysC* gene that leads to the desensitization of AK III activity (mutation *lysC10002*); the other is the partial O^c mutation for AK III (*lys-1100*).

The specific activity of three enzymes of the pathway has been measured in crude extracts of strains grown in the presence of an excess of lysine (in the case if strain Gif 106 lysine does inhibit growth, the inhibition being overcome by threonine plus methionine that were added in all cases).

Table III

Specific activities of some lysine biosynthetic enzymes in strain L-134

Strains	Growth conditions	Specific activity (nμmole/min/mg)		
		AK III	ASA-dehydrogenase	DAP-decarboxylase
Hfr H	minimal medium	22	630	6.7
	2×10^{-3} M-lysine	<1.5	320	2.7
	minimal medium	13.9	450	5
L-134	2×10^{-3} M-lysine	1.9	370	3.5

Enzyme assays were performed in cell-free crude extracts (ref. 22)

Table IV

DAP-decarboxylase activities of different strains in repression conditions

Strains	Mutations			Growth conditions	Specific activity (nμmole/min/mg)		
	1	2	3		AK III	ASA-dehydrogenase	DAP-decarboxylase
Gif 106	+	+	+	Min. medium (a)	37	1080	5.8
Gif 106				Repression (b)	1.5	350	0.3
106 G21	+	d	+	Repression (b)	1.5	340	0.9
ORA 101	0°	+	+	Repression (b)	23	330	0.3
ORA 103	0°	d	+	Repression (b)	23	340	5.1
106 M1	+	(c)	—	Repression (b)	0	340	0.3
106 M1				Lysine limitation (e)	0	4800	5.5

1: 0° mutation *lys-1100* leading to partially constitutive synthesis of AK III;

2: *lysC1002* mutation leading to densitization (d) of AK III activity;

3: mutation leading to the loss of AK III activity.

(a) The auxotrophic requirements of all the strains were met by addition of 0.5 mM L-isoleucine and 0.5 mM L-arginine.

(b) Repression conditions are 4 mM L-lysine (plus 5 mM DL-threonine, 2 mM DL-methionine, 2 mM L-isoleucine and 0.5 mM DL-arginine).

(c) This strain having lost its AK III activity, it is presumed that the allosteric lysine site has not been modified by the mutation.

(e) Growth in the presence of 75 μM DAP (plus threonine, methionine, isoleucine and arginine as above). In these conditions, growth is limited by the lysine pool at a concentration of about 10^9 bacteria/ml.

For isolation of the different mutant strains and for enzyme assays, see ref. 22.

It can be seen in Table IV that, when ASA-dehydrogenase synthesis is normally repressed in all the four strains, DAP-decarboxylase synthesis cannot totally be repressed in strain ORA 103 carrying both mutations.

These experiments suggest that DAP-decarboxylase synthesis is induced either by aspartyl-phosphate or by the AK III molecule itself.

Indeed, the synthesis of aspartyl-phosphate is possible only in strain ORA 103 grown in the presence of lysine that cannot inhibit or repress AK III. The possibility that this metabolite could be the inducer of the DAP-decarboxylase synthesis is, however, ruled out by the following facts. (i) In strain 106 M1 lacking all three aspartokinases, a derepression of DAP-decarboxylase synthesis may be observed in the presence of a limiting amount of lysine (Table IV). (ii) In a strain lacking AK I and AK III, but constitutive for AK II synthesis (Gif 881 [1]) and that must possess a high pool of aspartyl-phosphate even when the strain is grown in the presence of an excess of lysine, DAP-decarboxylase synthesis is normally repressible.

It appears that the synthesis of DAP-decarboxylase only occurs if (i) the AK III protein molecule is present (as in the parental strain in minimal medium or in the 0° mutant in the presence of an excess of lysine); and if (ii) it is under its non-inhibited conformation (as in the parental strain in minimal medium or in the desensitized mutant in the presence of an excess of lysine). Only strain ORA 103 that carries both mutations can fulfil these requirements in repression conditions. It is thus likely that the AK III enzyme is a positive inducer of DAP-decarboxylase synthesis, when it is under its non-inhibited allosteric conformation. The presence of lysine immediately leads to the allosteric transconformation, making the protein no longer capable to play its inducer role. It also represses, by an unknown mechanism, the synthesis of AK III inducer molecules that are diluted out during the subsequent growth of the cells.

We are currently using λ *lysA* phages and performing transcription experiments *in vitro* to ensure this regulatory role for AK III and its action at the transcriptional level.

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IMMUNOLOGICAL CROSS REACTIVITY OF FOUR ENZYMES INVOLVED IN THE BIOSYNTHETIC PATHWAY OF LYSINE, METHIONINE AND THREONINE IN *ESCHERICHIA COLI* K12*

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Summary. In *Escherichia coli* K12 the biosynthetic pathway of lysine, methionine and threonine is characterized by three isofunctional aspartokinases and two homoserine dehydrogenases. A single polypeptide chain carries the threonine-sensitive aspartokinase and homoserine dehydrogenase (AK I-HDH I), and a different polypeptide chain carries the methionine-repressible aspartokinase and homoserine dehydrogenase (AK II-HDH II). Immuno-adsorbants prepared with rabbit antibodies against AK I-HDH I bind the lysine-sensitive aspartokinase (AK III), the AK II-HDH II, and the homoserine kinase (HSK), an enzyme of the threonine biosynthetic pathway. Saturation of the immuno-adsorbant with AK I-HDH I results in a decreased binding capacity for the other enzymes. Displacement of bound AK III or HSK can be obtained with pure AK I-HDH I, showing that the affinity of the antibodies to homologous antigens is higher than to heterologous ones. Immuno-adsorbants prepared with anti-HSK antibodies show the same type of recognition: binding of the three aspartokinases and a capacity to displace the heterologous antigens bound. Accordingly, the same antibodies, implicated in the binding of the homologous antigen, bind the other enzymes. None of the other enzymes of the pathway, or the other kinases tested are recognized by the two immuno-adsorbants. It can be postulated that in *E. coli* K12, duplication of a common ancestor gene gave rise to the three aspartokinases and to the homoserine kinase; two of the genes coding for the aspartokinases fused with those coding for the homoserine dehydrogenases. Indicating that only few epitopes are shared by these enzymes, by conventional immuno-diffusion techniques no precipitation lines appeared with antibodies against AK I-HDH I and the other proteins.

Studies on protein evolution have been a field of expanding interest during the last ten years. This is due to the determination of the primary amino acid sequence of many proteins like haemoglobins, immunoglobulins, cytochrome C, ferredoxins, etc. [1].

Based on the knowledge of the amino acid sequence, evolutionary trees have been constructed relating the different organisms from which these proteins had been obtained [1].

Within a given species homologies in sequences have been found in proteins having identical or similar functions.

At a time when the amino acid sequences had not yet been known, a theory for the evolution of genes was proposed by HOROWITZ [2] and later by LEWIS [3]. With the acquisition of new data, this theory was expanded by OHNO [4, 5]. This theory states that evolution proceeds by gene duplication

* The substance of this paper was presented in a Colloquium on Regulation of Amino Acid Biosynthesis in Microorganisms, Ninth Meeting of the Federation of European Biochemical Societies, Budapest, August 25-30, 1974.

and further mutation in one of the redundant genes; this allows (a) the preservation of the original function and (b) the acquisition of a new function in the mutate gene. In the case of genes coding for proteins belonging to the same biosynthetic pathway, HOROWITZ suggested that these genes have a common origin [2, 6]; evolution proceeds backwards from the last enzyme of the "potential" biosynthetic pathway by tandem gene duplication and by functional differentiation.

Verification of the hypothesis of HOROWITZ requires determination of amino acid sequence of the proteins involved in a given biosynthetic pathway; in the absence of such data the use of immunological tools should, however, offer some information concerning the possible sequence homologies in these proteins.

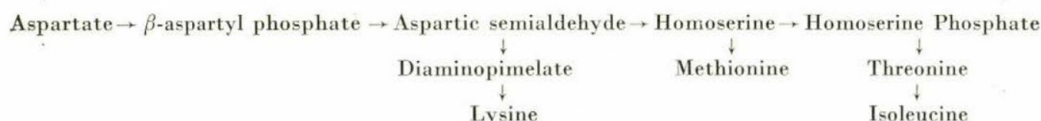


Fig. 1. Outline of biosynthetic pathway of the amino acids deriving all or part of their carbon from aspartic acid

The biosynthetic pathway of threonine, isoleucine, methionine and lysine of *E. coli* K12 (Fig. 1) is an interesting system for the study of protein evolution. This pathway is characterized by isofunctional and multifunctional enzymes: aspartokinase I, aspartokinase II and aspartokinase III catalyze the beta phosphorylation of aspartate; homoserine dehydrogenase I and homoserine dehydrogenase II catalyze the reduction of aspartate-semialdehyde to homoserine. Moreover, aspartokinase I and the homoserine dehydrogenase I, on the one hand, and aspartokinase II and homoserine dehydrogenase II on the other hand, are each carried by a single polypeptide chain [7, 8]. Table I summarizes the molecular weight of the polypeptide chains (protomers) of the enzymes involved from the native aspartokinase I-homoserine dehydrogenase I. Regulation of the synthesis and of the catalytic activity of the enzymes of this biosynthetic pathway have extensively been reviewed [9, 10].

A preliminary immunological study, using the precipitation technique in solid medium, failed to reveal immunological cross-reactivity between the three isofunctional aspartokinases [11].

The absence of immune precipitation cannot, however, be regarded as a proof of the different origin of two proteins: antibodies raised against the alpha chain of haemoglobin are unable to precipitate its beta chain [12]. Studies of the sequence have shown that the two polypeptide chains possess 40% sequence homology and derive from a common ancestor [13]. From these results it is clear that the immunoprecipitation techniques are not very sen-

sitive and that to achieve precipitation, a high homology in the amino acid sequence is required.

To avoid this pitfall, we have decided to use antibodies bound to Sepharose beads [14]; with this technique each molecule of antigen bound to a molecule of antibody will be retained on the column. The capacity of the immunoadsorbent for a given enzyme can thus be calculated from the total activity applied to the column and the amount found in the eluate [15].

Table I

Molecular weight and number of identical polypeptide chains of some enzymes of the biosynthetic pathway

Enzyme	Molecular weight of polypeptide chain	Number of subunits
Aspartokinase I-homoserine dehydrogenase I	86 000	4
Aspartokinase II-homoserine dehydrogenase II	86 000	2
Aspartokinase III	50 000	2
Aspartokinase I from an <i>ochre</i> mutant	48 000	4
Homoserine dehydrogenase I of the polypeptide fragment	55 000	2

We prepared immunoadsorbents with the antibodies obtained against the antigens aspartokinase I-homoserine dehydrogenase, the aspartokinase fragment of the non-sense mutant Gif-101 [16], the proteolytic fragment of the aspartokinase I-homoserine dehydrogenase I, carrying the homoserine dehydrogenase activity [17] and the homoserine kinase [18]. The antibodies were obtained by immunizing two rabbits for each pure antigen, with a total amount of 820 μ g of protein over a period of two months [19]. The antisera were then collected and the immunoglobulin fraction purified by ammonium sulphate precipitation; the purified fraction was coupled to cyanogen bromide activated Sepharose [14, 19].

The enzyme assays for the aspartokinase homoserine dehydrogenase [20], homoserine kinase [21], *N*-acetyl glutamo kinase [22] and aspartate semialdehyde dehydrogenase [23] have been described previously. Threonine synthetase assay takes advantage of an accessory threonine deaminase activity of this enzyme [24] and measures the formation of α -oxobutyrate.

The capacity of the different immunoadsorbents is expressed in nanomoles of subunits bound per ml of packed gel [15].

On an anti-aspartokinase I-homoserine dehydrogenase I immunoadsorbent are bound the "homologous" enzyme and the two fragments (Table II)

[15, 19, 25]. Three other enzymes are retained by this immunoadsorbent, viz., aspartokinase II-homoserine dehydrogenase II, aspartokinase III and homoserine kinase. The antisera used for preparation of the immunoadsorbent were, however, unable to precipitate these enzymes in solid medium [11] at different dilutions of the antigens and/or of the antisera. These antisera therefore do not contain antibodies selectively directed against each of the three enzymes due to a contamination by them of the aspartokinase I-homoserine dehydro-

Table II

Capacity of an anti-aspartokinase I-homoserine dehydrogenase I immunoadsorbent

Enzyme	Nanomoles of subunits per ml of gel
Aspartokinase I-homoserine dehydrogenase I	1.15
Aspartokinase I fragment	2.3
Homoserine dehydrogenase I fragment	1.15
Aspartokinase II-homoserine dehydrogenase II	0.60
Aspartokinase III	0.36
Homoserine kinase	0.30
Threonine synthetase	0
Threonine deaminase	0
Aspartate semialdehyde dehydrogenase	0
N-acetyl glutamo kinase	0

genase I used as antigen (very small amounts of contaminants would not have been detected by the techniques used to control the purity of the proteins, i.e. by electrophoresis on sodium dodecyl sulphate polyacrylamide gels or determination of the amino terminal residue).

The finding that the same molecule of antibody binds the "homologous" and the "heterologous" antigens is supported by experiments showing that saturation of the immunoadsorbant by the "homologous" protein decreases the subsequent binding of the other "heterologous" antigens.

Results reported in Table III show that an anti-homoserine kinase immunoadsorbant binds the heterologous enzymes aspartokinase I-homoserine dehydrogenase I and aspartokinase III. As it has been shown for the antisera directed against aspartokinase I-homoserine dehydrogenase I, the anti-homoserine kinase antibodies are unable to precipitate all the enzymes tested except homoserine kinase itself.

From the experiments reported above we may conclude that the three isofunctional aspartokinases and the homoserine kinase share antigenic determinants. These determinants are carried by the amino terminal part

Table III
Capacity of an anti-homoserine kinase immunoadsorbent

Enzyme	Nanomoles of subunits per ml of gel
Homoserine kinase	2
Aspartokinase I-homoserine dehydrogenase I	0.24
Aspartokinase III	0.14

of the aspartokinase I-homoserine dehydrogenase I molecule as it is shown in Table IV; only the antibodies obtained against the kinase fragment of the bifunctional enzyme are able to bind homoserine kinase and aspartokinase III.

Table IV
Capacity of the immunoadsorbent

Enzyme	Nanomoles of subunits per ml of gel	
	Anti-aspartokinase I fragment	Anti-homoserine dehydrogenase I fragment
Aspartokinase I fragment	1.0	0.12
Homoserine dehydrogenase fragment	0.1	0.43
Homoserine kinase	0.3	0

It is difficult to rule out the possibility that the cross-reacting antibodies are not directed against amino acid sequences or structures involved in the binding of the coenzyme (ATP) or of the aspartate and/or homoserine, or of the catalytic active site. Two sets of experimental data speak, however, against this hypothesis: (i) aspartokinase I is not inactivated after antibody binding; this suggests that the active site is not accessible to the antibodies; and (ii) *N*-acetyl glutamo kinase of *E. coli* K12, that uses ATP as substrate and catalyzes the same type of reaction, is not retained by the immunoadsorbant.

One is then left with the hypothesis of a common origin for the kinase part of these enzymes. From the study of aspartokinase I-homoserine dehydrogenase I it was postulated that this enzyme had arisen in *E. coli* K12 by gene fusion [17].

In contrast, in genera other than *Enterobacteriaceae*, the two reactions are catalyzed by two different polypeptide chains. Other examples have been reported of enzymes existing as separate entities in one genus and as multifunctional complexes in others (for a review, see [9]).

Experimental fusion of two genes has been described by YOUNG *et al.* [26] favouring the hypothesis of BONNER *et al.* [27], according to which gene fusion might be an important mechanism in the evolution of complex proteins.

If we then follow HOROWITZ's hypothesis [2, 6], we may speculate that homoserine kinase is the precursor of aspartokinase I, which would have originated by duplication of the original gene followed by further mutations. In favour of this we may cite the fact that aspartokinase has retained an intrinsic homoserine kinase activity and that homoserine inhibits the aspartokinase I activity of the wild type and of the aspartokinase fragment.

The evolution of the other two aspartokinases is not readily explained by HOROWITZ's hypothesis; they probably do not originate from "backward evolution"; gene duplication of the ancestor aspartokinase is more likely. More speculation is then required; it is plausible that the acquisition of a feedback regulation by the ancestor aspartokinase may have favoured the selection of isofunctional enzymes, thus allowing survival of the bacteria when an excess of the feedback effector is present in the medium.

How the fusion of aspartokinase and of the homoserine dehydrogenase took place remains subject to further speculations.

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REGULATION OF BRANCHED-CHAIN AMINO ACID BIOSYNTHESIS*

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Summary. Formation of branched-chain amino acids in microorganisms is controlled mainly by end-product inhibition of the enzyme action but also by repression of enzyme synthesis. The two mechanisms may be interrelated. Regulation of the metabolite flow by end-product inhibition is realized by the inhibition of threonine deaminase by isoleucine; inhibition of acetohydroxy acid synthetase by valine and inhibition of α -isopropylmalate synthetase by leucine. Acetohydroxy acid synthetase has a key position in the pathway, since the enzyme catalyzes not only the synthesis of acetolactate, the valine and isoleucine precursor from pyruvate, but also that of acetohydroxybutyrate, the isoleucine precursor from α -ketobutyrate and pyruvate. Quenching of valine pathway by valine would be expected to be accompanied by a quenching of the isoleucine pathway as well. Nevertheless, acetohydroxybutyrate is formed rather than acetolactate, since α -ketobutyrate has a greater affinity to the enzyme than pyruvate and the inhibitory effect of valine is directly proportional to substrate concentration. It could completely reverse the inhibition. Regulation on the physiological level of the pathways to the branched-chain amino acids occurs by the repression of the leucine and isoleucine-valine biosynthetic enzymes. A phenotypic derepression caused by valine was detectable in the case of the latter biosynthetic enzymes. The idea that aminoacyl tRNA formation is a necessary reaction in the formation of the "repressor" has been supported by numerous data. Indeed, some of the latest reports suggest that aminoacyl tRNA interaction with feedback sensitive enzyme may play an important role in the repression.

Valine, isoleucine and leucine as branched-chain amino acids have long been considered under a common heading. The basis for this grouping was their structural relationship. The metabolic relationship of the three branched-chain amino acids was observed in *Bacillus anthracis* by GLADSTONE [1] 35 years ago and he suggested that each of the amino acids may interfere with the utilization or the formation of the two others.

The application of isotopic tracer techniques and of mutant methodology then elucidated the biological and biochemical connections and it became clear that the three amino acids were indeed closely related as regards their biosynthesis [2].

The biosynthesis of the branched-chain amino acids was studied in detail by UMBARGER, ADELBURG, STRASSMANN and WEINHOUSE. The enzymes of the pathways were investigated in cell-free systems and some were partially purified. These observations led to the discovery of feed-back regulation.

* The substance of this paper was presented in a Colloquium on Regulation of Amino Acid Biosynthesis in Microorganisms, Ninth Meeting of the Federation of European Biochemical Societies, Budapest, August 25-30, 1974.

One of the earliest examples of universal validity concerning the control of metabolite flow by the end-product, namely the inhibition of threonine deaminase by isoleucine, was described by UMBARGER [3].

The formation of branched chain amino acids in microorganism is known to be controlled mainly by end-product inhibition of enzyme action but also by repression of enzyme synthesis. The two mechanisms may be interrelated.

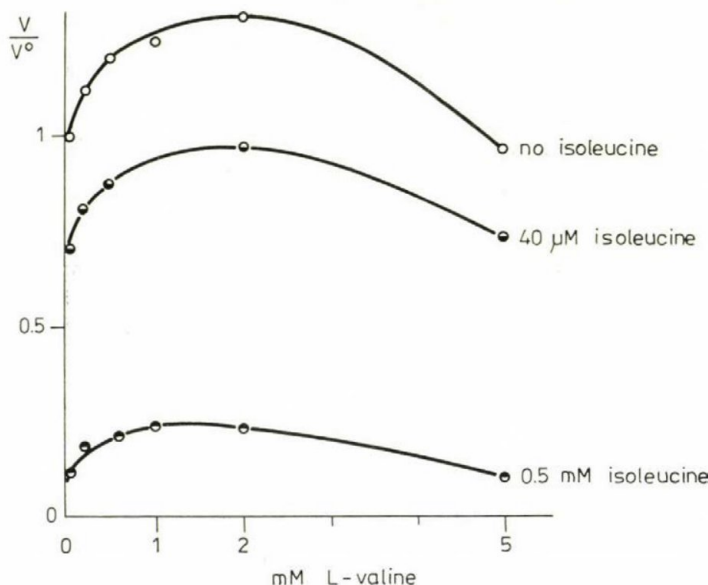


Fig. 1. Effect of valine on threonine deaminase. Reaction mixture: tris buffer (pH 7.8), 0.1 M ammonium chloride, 0.1 M; pyridoxal phosphate, 0.1 mM; L-threonine, 20 mM; protein, 0.25 mg/ml. Reaction time, 20 min at 28°C. The crude protein solution was extracted from ultrasonicated cells of *M. pellegrino*

The first and most effective short-term control is feed-back inhibition of the first enzyme, which is indispensable for the biosynthesis of the amino acid in question. In the branched-chain amino acid pathway, threonine deaminase, acetoxy acid synthetase and α -isopropylmalate synthetase are known to be sensitive to feed-back inhibition by isoleucine, valine and leucine, respectively [3-5].

The inhibition of threonine deaminase by isoleucine was not only the earliest example of the regulation by the end-product, but it has become one of the most interesting and widely accepted models of allosteric enzyme function, introduced by MONOD in collaboration with WYMAN and CHANGEUX [6-8].

The enzyme from *Escherichia coli* was investigated in detail by CHANGEUX [7]. He found that the binding sites of substrate and inhibitor were distinct and valine as an activator could stimulate the activity of threonine deaminase. Owing to difficulties in purifying the enzyme, the early observations were performed with crude extracts or with partially purified preparations.

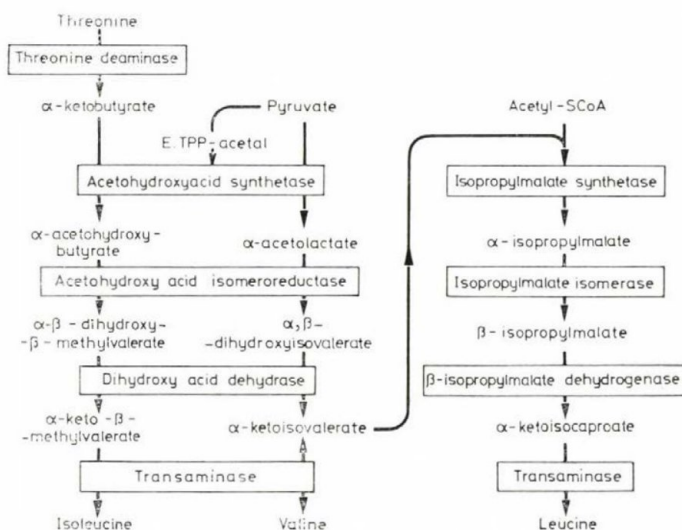


Fig. 2. Biosynthetic pathways leading to the branched-chain amino acids

More or less similar conclusions were obtained with cell-free extracts from other microorganisms, except for the activating effect of valine [9] (Fig. 1). The regulatory properties of threonine deaminase from *Mycobacterium pellegrino* were somewhat similar to the enzyme of *E. coli*. Valine in small amounts antagonized the inhibition caused by isoleucine and activated the enzyme in the absence of feed-back inhibitor. Valine could not, however, be activated by the desensitized threonine deaminase which was not inhibited by isoleucine.

Later, the enzyme was purified from *Salmonella typhi-murium* by MAEBA and SANWAL [10], and BURNS and ZARLENGO [11]. Some data refer to the possibility that threonine deaminase is composed of four identical subunits with a molecular weight of 200 000. The enzyme is probably a dimer of subunits which contains two identical peptides in such an arrangement that only one substrate site on each subunit is functional. It is now clear that the purified threonine deaminase of *S. typhi-murium* exhibits cooperative binding of substrate only in the presence of isoleucine [12].

The enzyme catalyzing the first common step in the biosynthesis of branched-chain amino acids — acetohydroxy acid synthetase (AHAs) — has a key position in the pathway (Fig. 2). This enzyme catalyzes not only the synthesis of acetolactate — the valine precursor from pyruvate — but also

that of acetohydroxy butyrate, the isoleucine precursor from α -ketobutyrate and pyruvate. AHAs could be inhibited by valine. Quenching of valine pathways by valine is accompanied by quenching of the isoleucine pathway, too.

Excess valine interfering with isoleucine biosynthesis could cause a complete inhibition of growth in *E. coli* K12. Isoleucine suspends the inhibitory effect of valine [13, 14], although the sensitivity of the enzyme to inhibition by valine is not the sole factor involved in the inhibition of growth. AHAs of *E. coli* W strain could be inhibited by valine as well as the enzyme

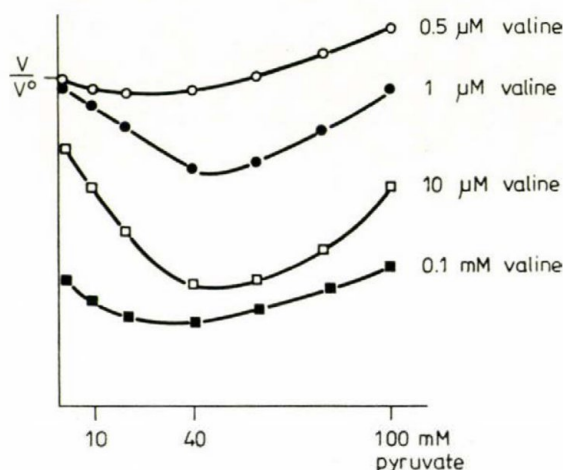


Fig. 3. Influence of pyruvate concentration on valine inhibition. AHAs was assayed according to UMBARGER and BROWN [37] in the presence of different amounts of valine

of strain K12, though valine does not inhibit the growth of *E. coli* W. It was therefore assumed that there were two physiologically distinct enzymes in the bacteria as STÖRMER demonstrated for *Aerobacter aerogenes*, but one of them proved to be feed-back sensitive [15].

We have investigated the enzyme obtained from *E. coli*, *Streptomyces rimosus*, *Pseudomonas aeruginosa*, *M. pellegrino* and *Candida tropicalis*. In cell-free system all of the enzymes were sensitive to valine. Valine does not, however, inhibit growth *in vivo* in minimal medium composed of ammonium sulphate and glucose. Investigating the properties of AHAs in detail, it was found that the valine sensitivity of this enzyme in cell-free system depends on the actual concentration of pyruvate (Fig. 3). In Fig. 3 the inhibitory effect of valine — as a quotient of the reaction velocity in the presence and absence of valine — is indicated versus the concentration of pyruvate. The synthesis of a cetolactate in cell-free system of *P. aeruginosa* was markedly inhibited by valine if the reaction mixture contained pyruvate at about 40 mM

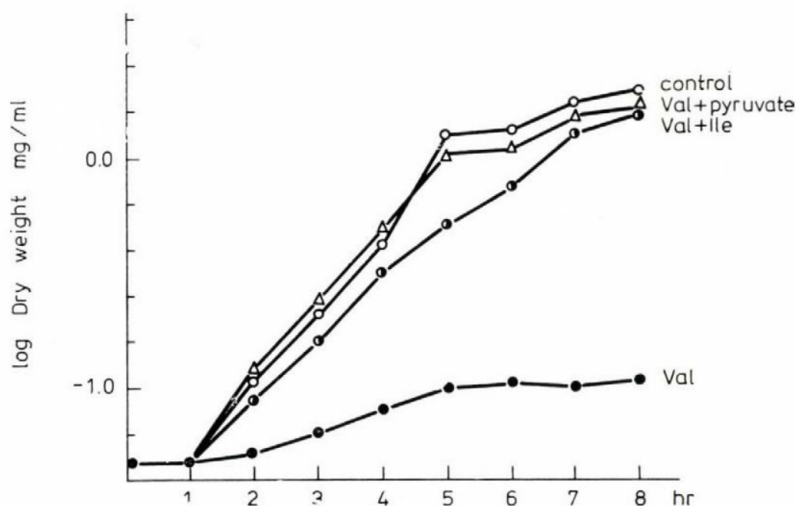


Fig. 4. Effect of valine on the growth of *P. aeruginosa*. ○—○ without addition; ●—● in the presence of 0.5 mg/ml L-valine; ○—● in the presence of 0.5 mg/ml L-valine and L-isoleucine; △—△ in the presence of 0.5 mg/ml L-valine and 2.0 mg/ml sodium pyruvate. The inoculum was grown overnight in broth at 37°C. Centrifuged and washed cells were inoculated into the medium (sodium-L-glutamate, 0.5%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; K_2HPO_4 , 0.7%; KH_2PO_4 , 0.3%; pH 7.0 before sterilization) supplemented as indicated above. Cultures were aerated at 37°C at the rate of 0.5 litre air per min

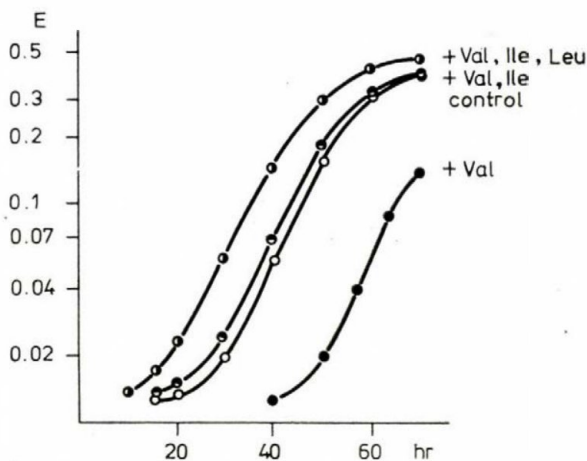


Fig. 5. Effect of valine on the growth of *M. pellegrino*. The medium contained asparagine, 0.4 g; glycine, 3.0 g; citric acid, 0.2 g; MgSO_4 , 0.05 g; thiamine, 0.04 g; Tween 80, 0.02 g, in 100 ml tapwater. Effect of valine and isoleucine was tested at 10^{-3} M concentration. ○—○ without addition; ○—● valine and isoleucine; ○—● valine, isoleucine and leucine; ●—● valine

concentration. The inhibitory effect of valine is connected with the pH and the temperature, too [16].

The inhibitory effect of valine on the growth of *Pseudomonas* could be detected in glutamate-containing glucose-free medium (Fig. 4). The existence of multiple forms of biosynthetic AHAs in *P. aeruginosa* has been suggested by VARGA and HORVÁTH [17]. The sensitive enzyme may be formed in the

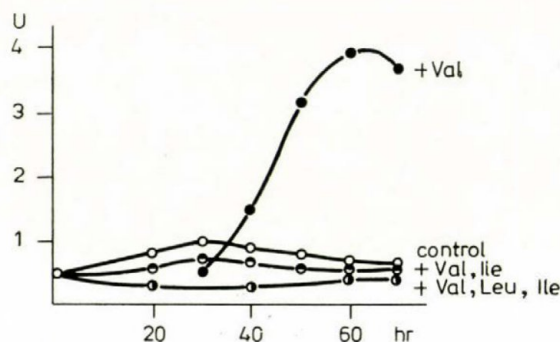


Fig. 6. Effect of valine on the level of AHAs. Cells were grown in shaker flasks at 28°C. ○—○ without addition; ◐—◐ valine and isoleucine; ●—● valine, isoleucine and leucine; ●—● valine

presence of pyruvate. Two years ago, evidence was presented independently by O'NEILL *et al.* [18] and BLATT *et al.* [19] that there are two forms of AHAs in *S. typhi-murium* and *E. coli*. One of them is sensitive while the other is resistant to inhibition by valine. Multiple forms of AHAs may be involved in the synthesis of isoleucine in the presence of excess valine. *E. coli* K12 may lack the ability to produce AHAs which is resistant to feed-back inhibition by valine, or may have resistant enzyme but in amounts inadequate for growth.

Using *M. pellegrino* it has been found that valine causes a prolonged log phase which could be shortened by valine (Figs 5 and 6). The high valine sensitivity of AHAs at first causes an isoleucine deficiency which may lead to a temporary inhibition of growth. Next, as a result of isoleucine deficiency, an increased synthesis of enzyme begins. As the enzyme concentration rises, sufficient isoleucine seems to be formed in the presence of valine, so that growth becomes possible [20].

RAMAKRISHNAN and ADELBERG [21] and FREUNDLICH and CLARKE [22] have shown that α -aminobutyrate mimics the effect of valine by inhibiting AHAs *in vitro* and also hinders the growth of *E. coli* W in minimal medium. In cultures of *E. coli* W we could observe the inhibition of growth caused by α -ketobutyrate or α -aminobutyrate (Fig. 7). In the presence of valine,

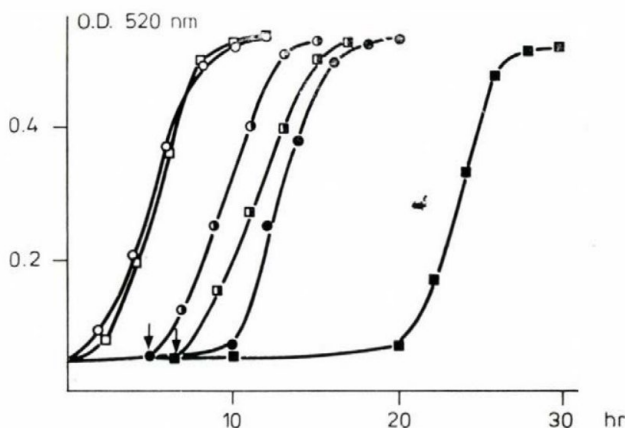


Fig. 7. Effect of α -ketobutyrate on the growth of *E. coli*. Cells were grown in minimal medium [38] in shaker flasks at 37°C. ○—○ control *E. coli* W; □—□ control *E. coli* W-110; ●—● *E. coli* W in the presence of 10 mM α -ketobutyrate; ■—■ *E. coli* W-110 in the presence of 10 mM α -ketobutyrate; ○—○ *E. coli* W in the presence of 10 mM α -ketobutyrate and 10 mM L-valine or 80 mM pyruvate added in the 5th hr; □—□ *E. coli* W-110 in the presence of 10 mM α -ketobutyrate and 10 mM L-valine or 80 mM pyruvate added in the 6th hr (arrows)

α -ketobutyrate had no inhibitory effect. Pyruvate or valine suspended the inhibitory effect of α -ketobutyrate. In whole cells, α -aminobutyrate was easily transformed to α -ketobutyrate by transaminase [23]. α -Ketobutyrate had an inhibitory effect on the formation of acetohydroxy acid though, like pyruvate, it is also a substrate of AHAs. The overall reaction takes place in two steps (Fig. 8). In the first step, TPP-acetal is formed from pyruvate and

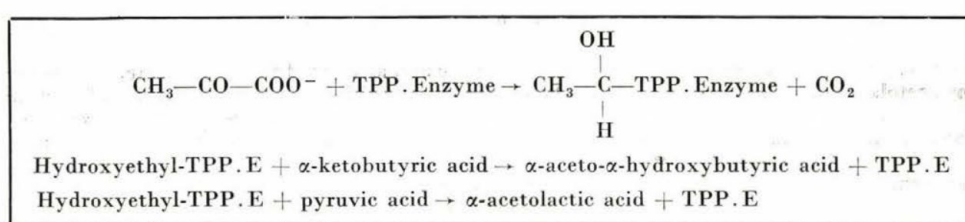


Fig. 8. Steps of AHAs activity

TPP and there is a competitive antagonism between pyruvate and α -ketobutyrate. In the second step of the reaction, acetohydroxy acid is formed from TPP-acetal and from pyruvate or α -ketobutyrate. We found that in different kinds of microorganisms acetohydroxy butyrate was formed rather than acetolactate since α -ketobutyrate had a greater affinity than pyruvate to the enzyme [24, 25] (Fig. 9).

The physiological importance of the different affinity of the enzyme to the two keto acids is essential for the life of microorganisms, because in the

cells the concentration of pyruvate is usually higher than the concentration of α -ketobutyrate (Fig. 10).

The third end-product sensitive enzyme in the branched-chain amino acid forming pathway is the first particular enzyme of leucine synthesis, α -isopropylmalate synthetase, which could be inhibited by leucine. It was highly purified from *Neurospora* and *Salmonella*. In both cases it was found that saturation of the enzyme by the two substrates was non-competitive [26].

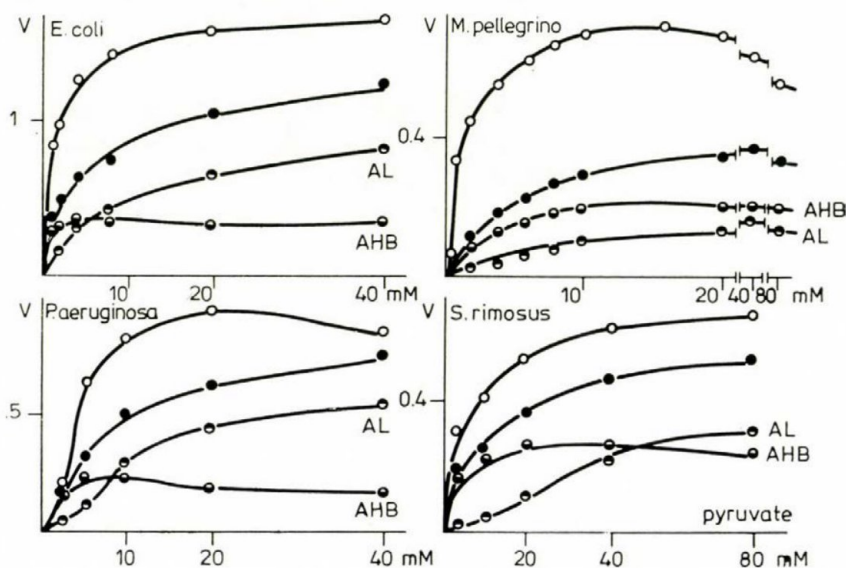


Fig. 9. Acetohydroxy acid formation in the presence and absence of α -ketobutyrate. Proportion of acetolactic acid and acetohydroxybutyric acid produced by four microorganisms. Reaction mixture: phosphate buffer (pH 8.0), 0.1 M; magnesium chloride, 0.01 M; thiamine pyrophosphate, 0.1 mM; flavine adenine dinucleotide, 0.01 mM; protein, 0.4 mg/ml. Reaction time: 20 min at 28°C. ○—○ amount of acetolactic acid formed without α -ketobutyrate; ●—● total amount of acetohydroxy acid formed in the presence of 1.0 mM α -ketobutyrate; ○—○ amount of acetohydroxy butyrate; ●—● amount of acetolactate

The regulation mechanism of the level of biosynthetic enzymes more or less conforms to the JACOB-MONOD model but it is generally accepted that this system is much more complicated than what a simple repressor operator model could describe.

The expression of gene cluster could be indicated by the amounts of the enzyme. The activity of all the specific enzymes required for branched-chain amino acid biosynthesis has to be assayed. Sometimes, owing to the different stability of the biosynthetic enzymes, their coordination is difficult

to demonstrate. For this reason not only the enzymological studies should be done carefully but the conclusions have also to be drawn with caution. The problem is further complicated by the hypothetical "multivalent repressor". Most observations were made by using *E. coli* and *S. typhi-murium*, but a number of experimental data have been obtained with other microorganism [20, 27].

The most interesting problem concerns the number of functional units at the level of gene expression and their mode of action. According to the facts presently accepted, in *Salmonella* there is a functional unit (leucine operon) of genes that defines the structure of the three specific enzymes required for

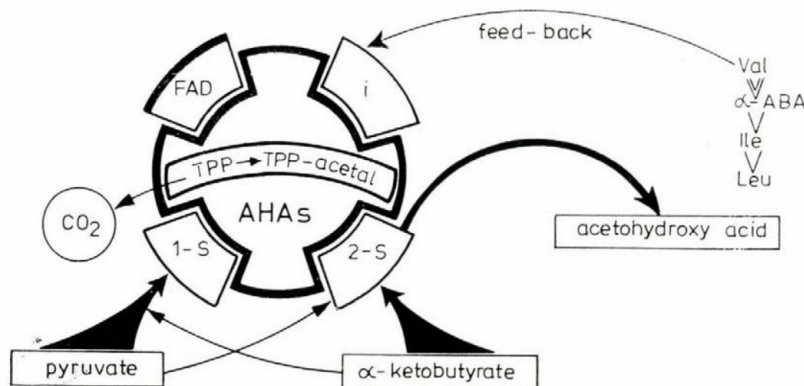


Fig. 10. Scheme of acetohydroxy acid production. 1-S represents the site of formation of TPP-acetal; 2-S represents the site of formation of AHA; i = symbol of valine (feed-back inhibitor); FAD (flavine adenine dinucleotide) activates the enzyme or protects it from inactivation

leucine biosynthesis. Repression and derepression of these enzymes seem to be coordinated. The mechanism of repression of the leucine operon is unclear because no mutants have been described which appear to lack the repressor of the classical model. On the other hand, GROSS found that in *Neurospora* isopropylmalate — a product of the first enzyme — was the inducer of the second and third enzymes in the pathway [28].

The physiological regulation of the repression of isoleucine-valine-forming enzymes is not clear. Experimental data unambiguously point to the existence of two distinct operators but there is no direct evidence for a third operator region.

Early experiments showed that the *ilv* gene cluster does not constitute a single operon. In *E. coli*, RAMAKRISHNAN and ADELBERG identified the distinct operator which regulates the formation of threonine deaminase, di-

hydroxy acid dehydrase and transaminase [21, 29]. The formation of AHAs and isomeroreductase is independent of the above-mentioned three isoleucine-valine forming enzymes. Most of the experimental data show that the formation of isomeroreductase is apparently coordinated with the formation of some of the AHAs. It might be as ARFIN *et al.* [30] have demonstrated that the production of AHA is the inducer of isomeroreductase. Like the pathway to leucine, the question of the regulation of the pathway to isoleucine and valine is an open one.

Earlier, the biosynthesis of branched-chain amino acids was looked upon as a negatively controlled system at the level of transcription. In many

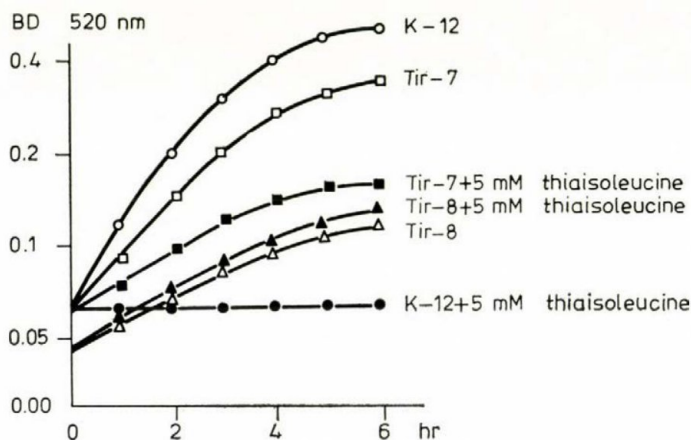


Fig. 11. Growth of K12 thiaisleucine resistant mutants in the presence and absence of thiaisleucine. ○—○ *E. coli* K12 grown in minimal medium; ●—● same, containing 5×10^{-3} M thiaisleucine; □—□ *E. coli* Tir-7 grown in minimal medium; ■—■ same, containing 5×10^{-3} M thiaisleucine; △—△ *E. coli* Tir-8 grown in minimal medium; ▲—▲ same, containing 5×10^{-3} M thiaisleucine

early works on the repression of biosynthetic systems it was assumed that the enzyme level was regulated by the availability of branched-chain amino acids. Total repression of the pathway could be detected in the presence of the three amino acids, isoleucine, valine and leucine. This simple concept then had to be modified because more and more experimental data suggested that aminoacyl-tRNA formation was a necessary reaction in the formation of the repressor. EIDLIC and NEIDHARDT [31, 32] showed that under conditions of a limited capacity to charge tRNA with valine, there was a derepression of *ilv* genes. On the other hand, FREUNDLICH reported [33] that α -amino- β -chlorobutyrate as an analogue of valine, which in itself was transferred to tRNA^{val}, led to the repression of the branched-chain amino acid-forming system, and

ALEXANDER *et al.* [34] observed that leucine must be activated by leucyl-tRNA synthetase to act in the repression of isoleucine, valine and leucine in *S. typhi-murium*. Subsequently, FREUNDLICH *et al.* [35] suggested an alternative explanation, namely that leucyl-tRNA synthetase is part of the repression mechanism.

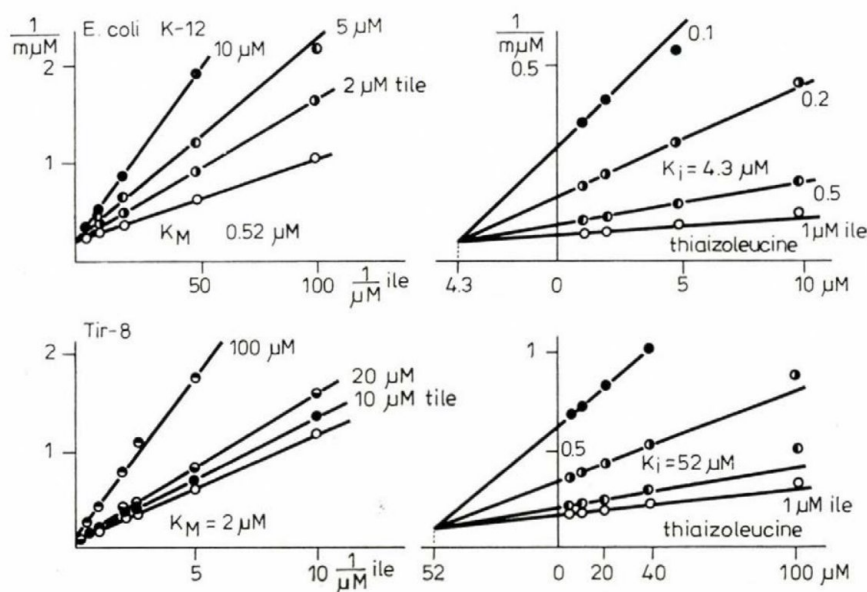


Fig. 12. Effect of thiaizoleucine on the charging of isoleucine-soluble RNA by isoleucyl-soluble RNA synthetase prepared from *E. coli* K-12 and Tir-8 thiaizoleucine resistant mutants

Our observation made in thiaizoleucine resistant mutants of *E. coli* K12 [36] supported the view that multivalent repression might involve the activated derivatives of the branched-chain amino acids. Thiaizoleucine and oxaisoleucine, analogous to isoleucine, by incorporating into the protein of the bacteria, could block the growth of wild-type *E. coli* K12. The inhibition of growth was reversed by isoleucine after a short lag phase. Isolating some mutant strains which were resistant against thiaizoleucine, it was found that the growth rate of the resistant strains was connected with the tolerated quantity of thiaizoleucine (Fig. 11). Extracts of these mutants exhibited a low isoleucyl-tRNA synthetase activity, which had a reduced affinity to isoleucine and even less to the analogous thiaizoleucine (Fig. 12). It seems, however, that the basis of the resistance of mutants was the derepression of their *ilv*

ADE operon (Table I). The thiaisleucine in the bacterial cells could be diluted by the overproduction of isoleucine. Some connections were found between the level of the derepressed *ilv* enzymes and the activity of isoleucyl-tRNA synthetase, and between the growth rate of the mutants and the tolerated quantity of the isoleucine analogue. The highest level of derepression of *ilv* enzymes could be detected in the case of strain Tir-8, which tolerated the largest quantity of thiaisleucine. This strain exhibited the lowest rate of growth and also the lowest level of isoleucyl-tRNA synthetase.

Table I

Levels of isoleucine and valine-forming enzymes in comparison with the specific activities of isoleucyl, leucyl and valyl soluble ribonucleic acid (sRNA) synthetase in cells grown in minimal medium

	TD	AHAs	IR	DAD	T-B	sRNA synthetase		
						isoleucyl	leucyl	valyl
<i>E. coli</i> K12								
parent strain	88	148	177	28	95	1.10	1.10	1.25
Tir-7 mutant	284	93	82	95	143	0.12	1.20	1.10
Tir-8 mutant	575	62	38	125	174	0.04	1.05	1.15
<i>E. coli</i> K12								
1 mM isoleucine, leucine and valine added	17	26	28	8	19	1.05	1.15	1.05

Specific activity is expressed as m μ moles per mg protein per min

In summary, the regulation of the flow of intermediates in the micro-organisms through the branched-chain amino acid biosynthetic pathway can be attained by (a) end-product inhibition of the key enzymes (feed-back sensitivity of threonine deaminase, acetohydroxy acid synthetase and isopropyl-malate synthetase); (b) regulation by substrate affinity (acetohydroxy acid synthetase); (c) end-product regulation by repression; and (d) substrate regulation by repression.

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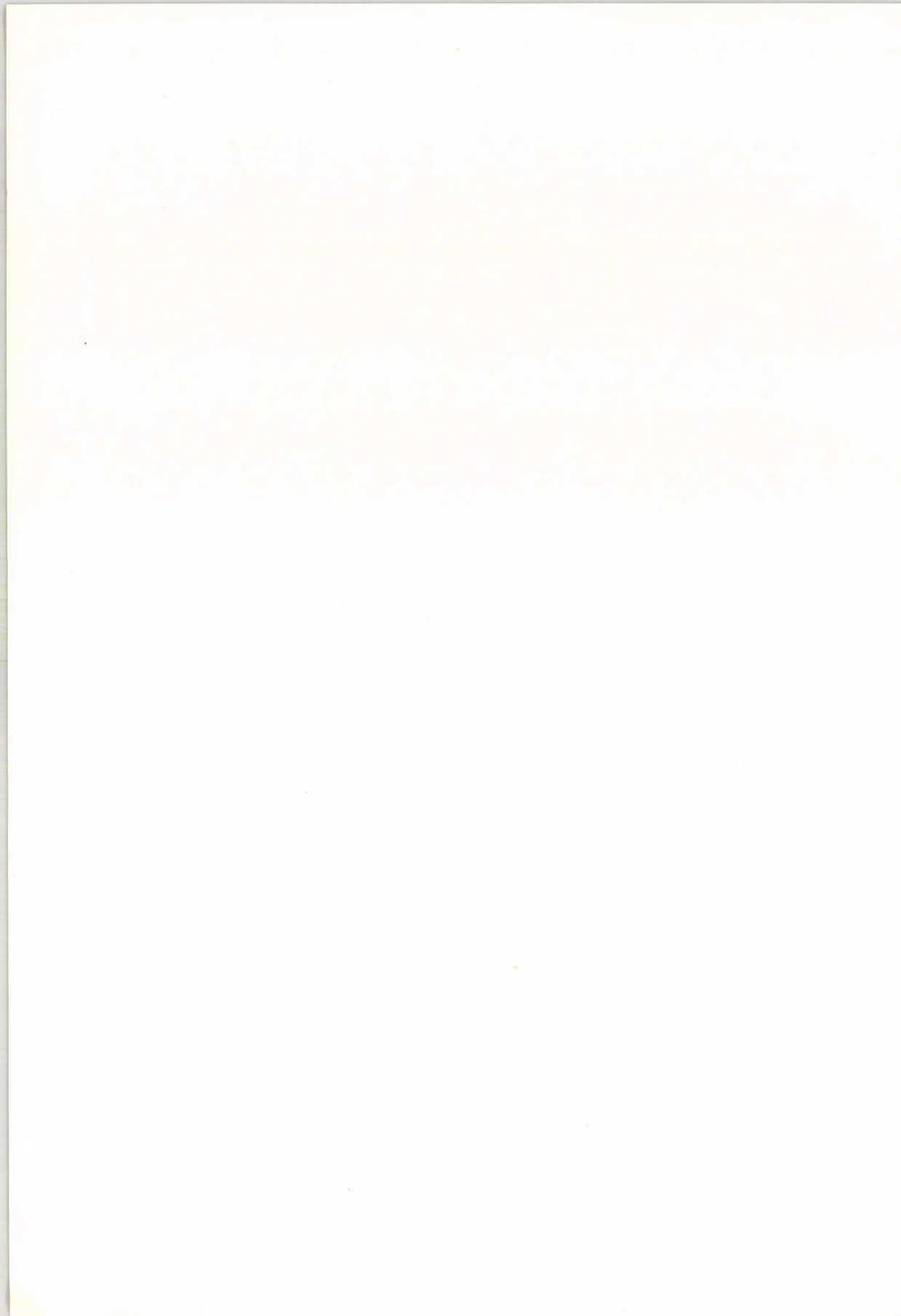
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GENE EXPRESSION OF THE HISTIDINE OPERON*

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Summary. Histidine biosynthesis in *Salmonella typhi-murium* and *Escherichia coli* is regulated through repression and feedback inhibition. Repression is triggered by the intracellular level of histidyl-tRNA whereas feedback inhibition is brought about by histidine itself, an allosteric inhibitor of the first biosynthetic enzyme. Several lines of evidence indicate that these two processes are interconnected and the first biosynthetic enzyme is itself a regulatory molecule for the *his* operon. The regulation seems to be exerted in a negative fashion, although a positive control cannot be excluded.

Histidine biosynthesis is carried out in ten subsequent steps each catalyzed by a specific enzyme. The genetics and the biochemistry of this system have been elucidated by the work of AMES, HARTMAN, GOLDBERGER and their associates [1]. The genes coding for each of the biosynthetic enzymes are clustered together on the chromosome of both *Salmonella typhi-murium* and *Escherichia coli*, and constitute the histidine (*his*) operon. A scheme of the *Salmonella* operon is presented in Fig. 1. Although less well studied, the *E. coli* system appears to be essentially the same.

The rate of synthesis of histidine is regulated by two different mechanisms: feedback inhibition and repression. The first mechanism consists in the inhibition of the first biosynthetic enzyme by the end product histidine [2]. In repression it is not the activity of the enzymes which is changed but their concentration in the cell. Under conditions of histidine limitation, the cell produces biosynthetic enzymes at a faster rate (derepression) and therefore the intracellular level of the enzymes increases. When, however, histidine is in excess, the rate of synthesis falls abruptly and the enzyme concentration decreases to the background value (repression) [3].

Derepression and repression of the *his* operon are experienced by all structural genes as a whole: gene expression is in fact coordinated, the *his* operon reacting as a unit in response to the available level of histidine of the organism [4]. Mutations to the left of the *hisG* gene result in a cis-dominant

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Fig. 1. Map of the histidine operon of *S. typhi-murium*. The numbers at the top indicate the metabolic steps in the biosynthetic pathway, which are catalyzed by the individual enzymes. The second line shows the symbols of the structural genes. To the left of the *G* gene is located the operator-promoter region (not shown in the figure). Below are shown the complementation groups and some of the point and deletion mutants. (From reference [1])

constitutive expression of the histidine biosynthetic enzymes and define the operator region [5]. Promoter mutants have also been found and mapped within the operator [6, 7]. An accurate genetic analysis of the operator-promoter region shows that promoter mutants are sandwiched among operator constitutives and suggests a direct role of the DNA structure in the regulation of gene expression [7].

Histidine regulatory mutants which map outside of the *his* operon have been isolated and studied in great detail. Five distinct classes have been identified and their location on the chromosome is shown in Fig. 2. The properties of these mutants are summarized in Table I.

Table I
Properties of his regulatory mutants of S. typhi-murium

Locus	Gene product
<i>hisR</i>	tRNA ^{his}
<i>hisS</i>	histidyl-tRNA ^{his} synthetase
<i>hisT</i>	enzyme converting uridine into pseudouridine in the anticodon loop of tRNA ^{his} (pseudo-uridilate synthetase)
<i>hisU, hisW</i>	other enzymes involved in the maturation of tRNA ^{his}

For specific references see [1]

All mutants have elevated levels of histidine biosynthetic enzymes. These data have been obtained by AMES and his group (see [1] for references) who have analyzed a large number of mutants of *Salmonella typhi-murium*. Analogous mutants have also been found in *E. coli* (BLASI and SBORDONE, unpublished).

From the study of the regulatory mutants, one concept becomes unquestionable: whatever the mechanism, it is clear that histidyl RNA must play a role in the regulation of gene expression. LEWIS and AMES [8t] analyzing the histidyl-tRNA^{his} content of several constitutive mutants have come to the conclusion that the level of constitutivity is inversely proportional to the intracellular level of charged tRNA^{his}, and that the control is exerted in a negative fashion.

While the importance of histidyl-tRNA^{his} in regulating the expression of the *his* operon is clearly established, the mechanism through which repression is exerted is not clear; nor is it clear whether the charged tRNA acts by itself or as a part of a more complex repression system. Most investigators feel that histidyl-tRNA^{his} might be a co-repressor; genetic data have, however, failed to reveal any typical "repressor protein". Biochemical investigations by GOLDBERGER and his associates strongly suggest that the product of the

first structural gene (*hisG*), which is the first biosynthetic enzyme, might itself be involved in regulation of the *his* operon. First, the kinetics of repression of some *hisG* mutants are strongly altered, and in some cases repression cannot be achieved by the use of the histidine analogue triazole-alanine [9]. These mutants are either feedback-resistant mutants in *hisG* or *hisG* amber mutants, but histidine is able to repress normally in every cases. On the other hand, an absolute, low-level constitutive mutant has been isolated which maps in *hisG* [10]. This mutant is feedback resistant, has a normal catalytic activity

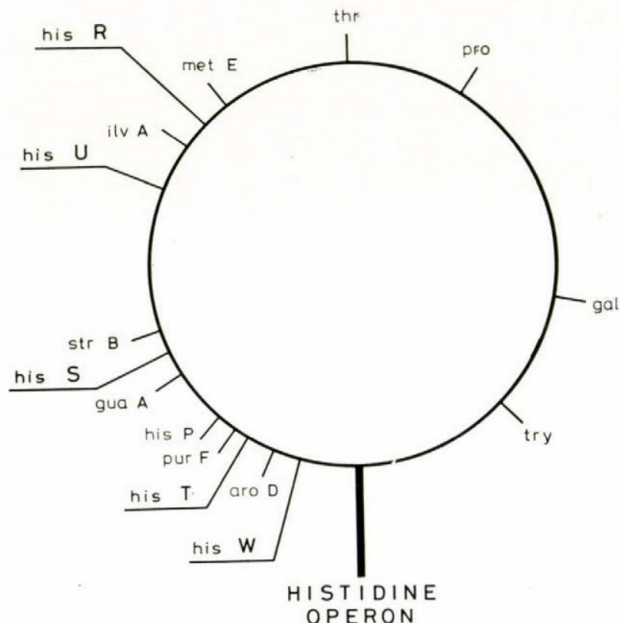


Fig. 2. Schematic map of the chromosome of *S. typhi-murium* showing the location of the *his* regulatory mutants and some genetic markers. See Table I for the explanation of the various classes of *his* regulatory mutants

and its altered constitutivity is relieved when a normal copy of the *hisG* gene is also present in the cell. Therefore the regulatory role of *G* enzyme is exerted in *trans*.

The product of the first structural gene is the feedback-sensitive enzyme phosphoribosyl-ATP synthetase, which will be abbreviated as *G*-enzyme. The enzyme condenses ATP and phosphoribosyl pyrophosphate to form phosphoribosyl-ATP, liberating pyrophosphate: $\text{PRPP} + \text{ATP} \rightarrow \text{PRATP} + \text{PP}_i$. This enzyme is inhibited by histidine [2, 11, 12] with a K_i of approximately 7×10^{-5} M [13].

If *G* enzyme is directly involved in repression of the *his* operon, since histidyl-tRNA^{his} too is involved in the regulation of this operon, one might

expect that the two molecules are the constituents of a "repressor complex", and therefore that *G* enzyme and histidyl tRNA^{his} might interact *in vitro*. This prediction has been verified. *G* enzyme binds histidyl-tRNA from a mixture of tRNA with a binding constant of about 10^{-7} — 10^{-8} M [14]. The binding is inhibited by the substrates and takes place at a site different from both the active site and the allosteric site [15]. The specificity for the charged form of tRNA is very high [16], in keeping with the data of LEWIS and AMES [8] and the above-mentioned prediction of a "repressor complex".

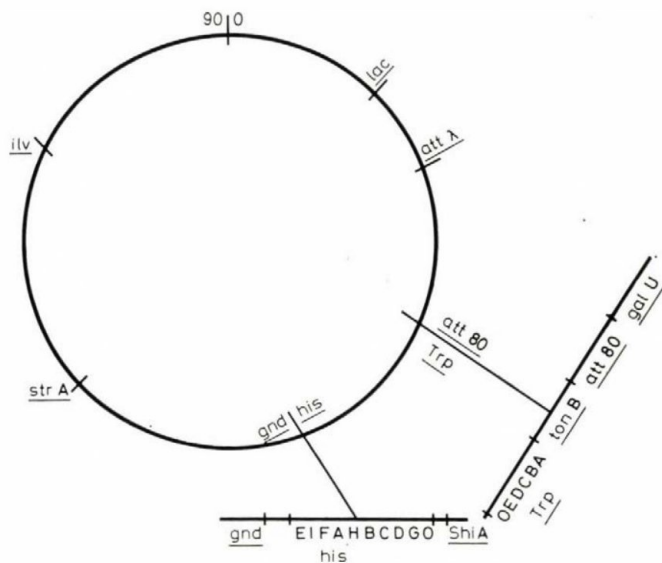


Fig. 3. Scheme of the *E. coli* chromosome showing the location of the *his* operon and of the *tonB* locus. *Att80* is the attachment site for phage $\theta 80$. Transposition of *his* into *tonB* has been necessary as a pre-requisite to the isolation of a *his* transducing phage

If the *G* enzyme has a direct role in the expression of the *his* operon, one should be able to verify this point *in vitro*. Regulation of the *his* operon is exerted at the transcriptional level, as shown by the increase in *his* mRNA during derepression [17, 25], and in the constitutive mutants of both *Salmonella* (KASAI and HARTMAN, unpublished) and *E. coli* (COVELLI and BLASI, unpublished). Therefore we have attempted to demonstrate an effect of the *G* enzyme on *in vitro* transcription of the *his* operon.

The first step in establishing an *in vitro* system for mRNA and enzyme synthesis consists in the appropriate choice of the template. An appropriate template could be a specialized transducing phage for the *his* operon. We have constructed such a phage using a technique originally employed in the isolation of a phage transducing the *ara* operon [18]. Fig. 3 shows a map of the *E. coli* chromosome. The *his* operon is located quite far away from the attach-

ment sites (*att*) of either of the two temperate phages of *E. coli*, Ø80 and lambda. Because the chance of a spontaneous event leading to the formation of a transducing phage depends on the distance between the phage and the bacterial genes [19], such an event cannot be expected to take place in this case. The *his* operon had therefore to be transposed into a site closer to the attachment site of a temperate phage. We chose to transpose *his* close to *att80*, the site for phage Ø80, located close to the tryptophan operon (*trp*). This was done in the following way: a temperature sensitive $F'_{T.S.}his^+$ episome was conjugated with a strain carrying a complete deletion of the *his* operon. The episome was forced to integrate into the locus *tonB* (adjacent to *att80*) by a double selection: temperature resistance and inactivation of *tonB* (inactivation of *tonB* confers resistance to phage T1, or Ø80*vir* and colicins) [19, 20]. Fig. 4

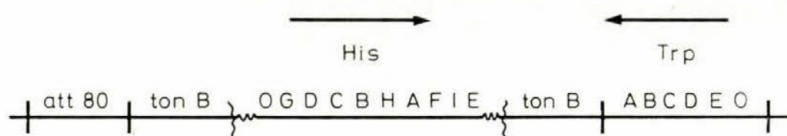


Fig. 4. Schematic map showing the insertion of the *his* operon in *tonB* and its orientation in respect to the *trp* operon. This chromosome was lysogenized with Ø80 imm^{λ} at *att80* and from this was isolated the transducing phage Ø80 $imm^{\lambda} dhis$ [21]

shows the results of the integration of $F'his$ into *tonB*. The *his* genes are now located close to *att80*. This direct transposition strain was then lysogenized with phage Ø80 h carrying λ immunity with a temperature sensitive repressor. Heat induction of this lysogen produced a lysate, and this was used to transduce to His^+ a complete deletion of *his*. Transducants arose at a frequency of about 10^{-9} and could be shown to be double lysogens, carrying both a wild type and a *his* transducing phage. This phage, Ø80 $imm^{\lambda} dhis$, has been analyzed in detail both by marker rescue and by deletion analysis in the prophage state. Infection of a single defective lysogen with Ø80 amber mutants in different genes of the Ø80 genome provided evidence for defectiveness of the transducing phage. All late genes, except the first three head genes, are missing in the transducing phage, and are substituted by bacterial ones. These include all structural *his* genes since the phage can transduce a complete deletion of *his*, and also carries the structural gene for gluconate-6-phosphate dehydrogenase (*gnd*). The orientation of the *his* operon in respect to the adjacent *trp* operon and to the other phage genes was determined by isolating deletions which start on one side in the *trp* operon and end on the other side at different levels into the *his* operon. Analyzing the residual *his* genes in these deletions we have concluded that (i) the *his* genes are located between the immunity region and *trp* operon; (ii) gene *gnd* lies between *his* and *trp*; (iii) the orientation of the *his* operon is opposite to that of the *trp* operon. There-

fore the situation can be summarized as follows:

gal U-a tt-phage imm (cI)-his (OGD...E)-gnd-att-trp (ABCDEO)

From these data one conclusion can be drawn, which is relevant to the use of this phage as a template in an *in vitro* system: the direction of transcription being away from the operator gene, the phage DNA strand which codes for the *his* operon mRNA must be the same strand that would code for the late phage genes, i.e. the heavy, or "R" strand. Fig. 5 shows a schematic view of the map of $\phi 80$ *imm⁺ dhis*, in which the two strands of the DNA have been drawn separately in order to show the direction of transcription of several parts of the genome [21, 22].

Gene expression can be studied *in vitro* either by means of a purified transcription system, or through a cell-free system for enzyme synthesis. Since

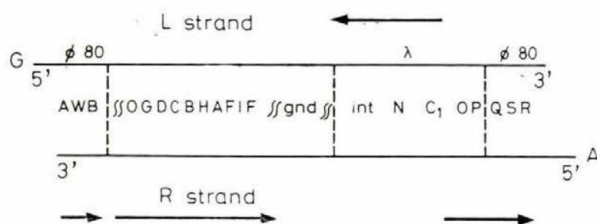


Fig. 5. Genetic map of $\phi 80$ *imm⁺ dhis* DNA with the two strands separated. The "R" strand (which in λ has an A in the 5' terminal position) is the template for *his* operon transcription

the two possible regulatory molecules of the *his* operon are either an integral part of the protein-synthesizing system, histidyl-tRNA, or a product of the cell-free synthesis itself (*G* enzyme), we decided to use the purified *in vitro* transcription system.

The DNA of the *his* transducing phage was therefore incubated with purified RNA polymerase from *E. coli* K12, in the presence of the four nucleo-

Table II

Hybridization analysis of the RNA synthesized *in vitro* on $\phi 80$ *imm⁺ dhis* DNA template

	cpm $\times 10^{-4}$	Per cent
Total incorporation	2.45	100
Hybridized to $\phi 80$ <i>imm⁺ dhis</i> DNA	1.98	80.8
Hybridized to $\phi 80$ <i>imm⁺ L</i> DNA	1.37	55.9
Hybridized to $\phi 80$ <i>imm⁺ dhis</i> "R"	1.12	45.7
ΔR	0.37	15.1
Competed by cold <i>his</i> -rich RNA	0.31	12.6

See ref. [23, 24] for methods of synthesis and of hybridization of RNA. All data refer to conditions of saturating DNA concentration, except the competition data in which the $[^3H]$ RNA was in excess.

side triphosphates at appropriate KCl and $MgCl_2$ concentrations. In general, 300 μM triphosphates, 60 μM KCl and 10 mM $MgCl_2$ were employed; [3H]UTP was used as label.

His RNA synthesized in this *in vitro* system can be assayed by hybridization to the separated strands of $\phi 80 imm^+ dhis$ and $\phi 80 imm^+$ DNA. The difference of counts hybridized to the R strands contains the *his* RNA. This can also be determined more precisely by hybridization competition, using cold RNA extracted from cells derepressed for the *his* operon. Table II shows an analysis of the RNA product made *in vitro* on $\phi 80 imm^+ dhis$ DNA template [23]. The RNA complementary to the bacterial region of the template accounts for about 30% of the total. Of this, about half is complementary to the R strand and can totally be competed out by a *his*-rich RNA preparation (prepared by a strain constitutive for the *his* operon).

Table III
Effect of G enzyme on in vitro transcription of the his operon

	Total RNA (cpm) $\times 10^{-6}$	<i>his</i> RNA cpm $\times 10^{-6}$	Per cent
—G	1.47	0.198	13
+G	1.36	0.004	0.3

See ref. [23, 24] for methods of synthesis and detection of *his* mRNA. *G* enzyme was pure by all common criteria of purity.

Table III shows the effect of *G* enzyme on the synthesis of *his* RNA *in vitro* by *E. coli* RNA polymerase at a molar ratio *G*/DNA of about 70 [24]. *G* enzyme blocks completely and specifically *his* RNA synthesis.

From these data two conclusions may be drawn. 1. Transcription of *his* operon of *E. coli* carried by the transducing phage takes place in a purified system. Therefore at this time one does not need to consider any kind of positive control in the *his* operon. Since the *his* operon of *S. typhi-murium* is not transcribed *in vitro* by a purified *E. coli* RNA polymerase [25], we cannot exclude that some positive control is acting in the system, but we have been unable to find any evidence for it. 2. In our *in vitro* system the *G* enzyme is acting as a repressor. More accurate analysis (DI NOCERA, AVITABILE and BLASI, unpublished) has further confirmed the absolute specificity of this effect. However, we do not find it necessary to add histidyl-tRNA to obtain the action from the *G* enzyme. Further work is therefore needed to understand the exact mechanism.

Since *S. typhi-murium his* DNA is not transcribed *in vitro* by the *E. coli* RNA polymerase [25], the effect of *G* enzyme cannot be studied. It has, however, been established that *G* enzyme does bind to the DNA of a phage that

carries the *his* operon, while not to the parental phage. Moreover, this binding is abolished by a θ^c mutation (MEYERS, VOGEL and GOLDBERGER, to be published).

In conclusion, it appears that some differences could exist in the regulation of two related species like *S. typhi-murium* and *E. coli* in the way their *his* operon is regulated. The first enzyme for histidine biosynthesis, specified by the first structural gene *hisG*, appears to act as a negative regulator in the system, although not all crucial details of its action have been clarified. The *his* operon appears therefore to be a very interesting example of autogenous regulation [26] in which the product of an operon is able to control the expression of the very same operon. To elucidate the role of histidyl-tRNA in *his* operon expression, studies are in progress.

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REGULATION OF AROMATIC AMINO ACID BIOSYNTHESIS IN MICROORGANISMS*

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Summary. Aromatic amino acid biosynthesis, chorismate mutase, prephenate dehydratase, prephenate dehydrogenase, anthranilic acid synthetase and 3-deoxy-D-arabinose heptulosonic acid 7-phosphate synthetase activity and inhibition in various microorganisms have been discussed.

The biosynthesis of aromatic amino acids starts with intermediates of the carbohydrate metabolism. The first step in the main pathway is the condensation of phosphoenolpyruvic acid with erythrose-4-phosphate to form 3-deoxyarabinoheptulosonic acid 7-phosphate. The enzyme that catalyzes this reaction is DAHP synthetase. In many microorganisms this step is catalyzed by isozymes, which all catalyze the same reaction and are all typically inhibited by the end products of the branched biosynthesis chain [1]. JENSEN and NESTER have found that in *Bacillus subtilis* the first step in the main branch is inhibited allosterically by prephenic acid and by chorismic acid, but not by the terminal amino acids [2–5]. In this case there exists only one DAHP synthetase. The second step of the main pathway is the conversion of 3-deoxyarabinoheptulosonic acid 7-phosphate into 5-dehydroquinic acid. The enzyme mediating this transaction, 5-dehydroquinic acid synthetase, works only when strongly activated by Co^{2+} [6]. Chorismic acid closes the main step of aromatic amino acid biosynthesis and from this point we have to discuss the enzymes which are able to convert chorismic acid.

The conversion of chorismic acid into prephenic acid is catalyzed by chorismate mutase. Prephenic acid is the common starting material for the biosynthesis of phenylalanine and tyrosine. Prephenic acid is aromatized enzymatically by prephenate dehydratase. The conversion of prephenic acid into p-hydroxyphenyl-pyruvic acid is catalyzed by prephenate dehydrogenase. This reaction requires NAD as a cofactor.

* The substance of this paper was presented in a Colloquium on Regulation of Amino Acid Biosynthesis in Microorganisms, Ninth Meeting of the Federation of European Biochemical Societies, Budapest, August 25–30, 1974.

In tryptophan biosynthesis the first step is the conversion of chorismic acid into anthranilic acid. This step is catalyzed by anthranilic acid synthetase.

In the last few years we have studied the mentioned enzymes in *Saccharomyces cerevisiae* [7-9], *Claviceps paspali* [10-13] and in *Streptomyces* strains [14-17]. The chorismate mutase of *Streptomyces aureofaciens* Tü 24 has been purified 2200-fold and the preparation is homogeneous by the criterion of polyacrylamide gel electrophoresis. To obtain a homogeneous preparation it is essential to treat the enzyme solutions with phenyl methane-sulphonyl fluoride, an inhibitor of serine proteases, after each purification step. Chorismate mutase has a molecular weight of 51 000 as determined by sucrose gradient centrifugation or of 63 000 as determined by Sephadex gel chromatography. The reason for the discrepancy is not known, but similar differences have been reported for chorismate mutase-prephenate dehydratase from *Salmonella typhi-murium* [18]. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis reveals a single protein band, the molecular weight of which is estimated to be 14 500. The native enzyme thus consists of at least three polypeptides of identical or almost identical molecular weight. These subunits are not linked together by disulphide bridges, because it is not necessary to use a reducing agent like β -mercaptoethanol during subunit dissociation and separation. The activity of the enzyme does not depend on the presence of metal ions, nor are sulfhydryl groups present, which are essential for catalytic activity. Heat inactivation of chorismate mutase, which occurs above 60 °C is reversible. The enzyme activity can be restored even when chorismate mutase is treated at the temperature of a boiling water bath for 15 min. Heat-denatured and renatured enzymes showed the same Michaelis constant (K_m of 5.3×10^{-4} M) and the same molecular weight as the native enzyme. L-phenylalanine, L-tyrosine, L-tryptophan, and intermediates of the aromatic amino acid pathway were tested as potential modifiers of chorismate mutase activity. The activity of the enzyme was inhibited by none of these substances. Chorismate mutase was not repressed in cells grown in minimal medium supplemented with L-phenylalanine, L-tyrosine, or L-tryptophan. These properties of chorismate mutase from *S. aureofaciens* [19] are very similar to those of chorismate mutase from *Streptomyces venezuelae* [16]. Chorismate mutase of *S. aureofaciens* is not associated with prephenate dehydrogenase, prephenate dehydratase, or anthranilate synthetase activities and appears to be a single enzyme. In this way it resembles the chorismate mutase of eukaryotes. But in contrast to the enzymes from eukaryotes, chorismate mutase from *S. aureofaciens* is not modified by the intermediates and products of aromatic amino acid biosynthesis.

Concerning these findings and the properties of DAHP synthetase, it appears that the whole route of phenylalanine and tyrosine biosynthesis is not influenced by its end products in *S. aureofaciens*. The level of tryptophan

concentration acts as the signal which increases or decreases the rate of synthesis of aromatic amino acids. DAHP synthetase from *S. aureofaciens* is inhibited by tryptophan (Fig. 1).

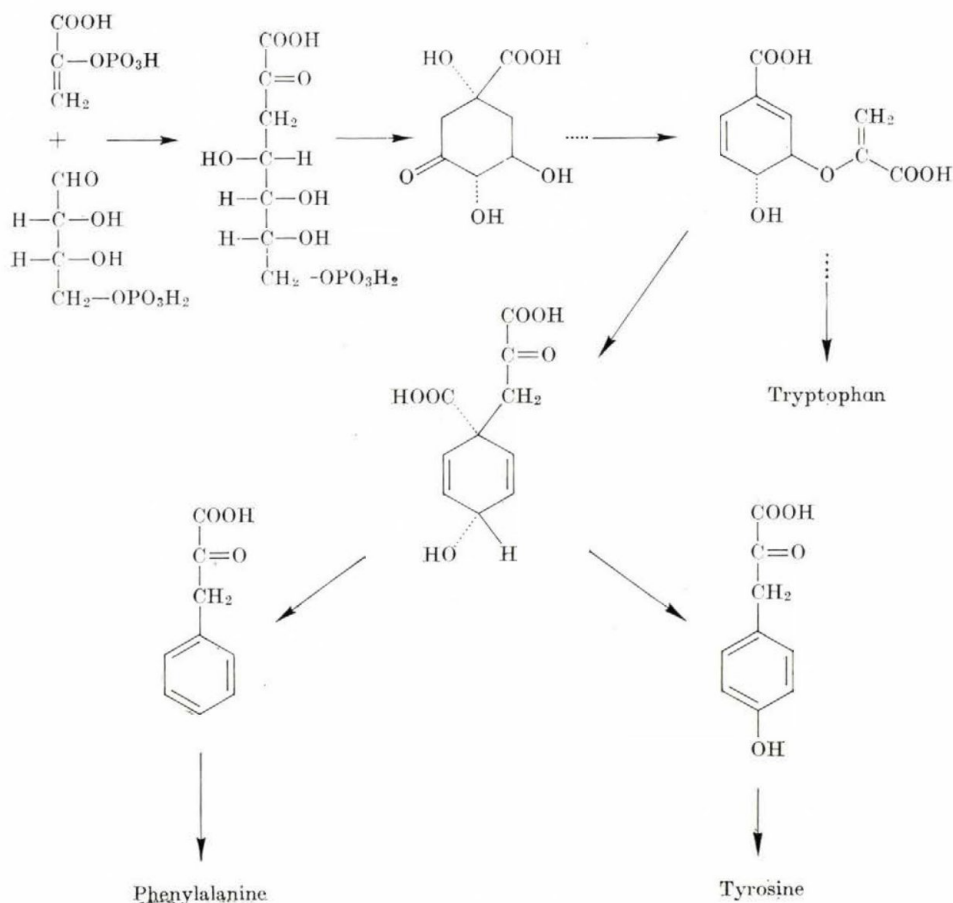


Fig. 1. Route of phenylalanine and tyrosine biosynthesis

The mode of action of the postemergence herbicide *N*-phosphonomethylglycine has been examined by JAWORSKI [20]. This herbicide has proved particularly promising for the destruction of resistant perennial weeds such as couch grass and bindweed. From growth experiments with the plants *Lemna gibba* and *Rhizobium japonicum*, JAWORSKI concluded that the effect of the compound was due to an inhibition of the biosynthesis of aromatic amino acids.

The inhibitory action of *N*-phosphonomethylglycine could be reversed by the addition of aromatic amino acids. JAWORSKI suggested that this com-

pound may inhibit or repress chorismate mutase and/or prephenate dehydratase.

Inhibition *in vitro* of these enzymes from *E. coli* B could not be observed. Growth of *E. coli* B was completely inhibited by *N*-phosphonomethylglycine at a concentration of 2×10^{-3} M. This inhibitory effect was reduced by the addition of phenylalanine. Tyrosine at the same concentration counteracted the inhibition to a lesser extent. The two amino acids administered simultaneously had an increased effect. The effect of tryptophan was slight, but nevertheless significant. The action of *N*-phosphonomethylglycine was completely eliminated by the addition of a mixture of all three amino acids. No effect was observed with histidine, valine or leucine. *N*-phosphonomethylglycine ($\text{H}_2\text{O}_3\text{P}-\text{CH}_2-\text{NH}-\text{CH}_2-\text{COOH}$) inhibited DAHP synthetase and 5-dehydroquinic acid synthetase. Both inhibitory effects were blocked by the addition of Co^{2+} [21].

5-Amino-4-chloro-2-phenyl-3(2 H)-pyridazinone (pyrazone) is the active ingredient in the herbicide Pyramin[®], which is used for the control of broad-leef weeds in sugar beets and table beets. Bacteria capable of growth on pyrazone as the sole source of carbon have been isolated from soil [22]. Cobalamine was required as a growth factor in a pyrazone-mineral salt medium. The bacteria are osmotically sensitive. Growth is not observed in media with a concentration of ingredients exceeding 1%. The bacteria do not grow with the usual carbon sources, such as glucose, fructose, glycerol, etc. Pyrazone is metabolized to the dephenylated compound [23]. Four compounds could be isolated from the medium of the growing bacteria. A pathway for the degradation of this herbicide could be proposed (Fig. 2).

The bacteria could not be classified taxonomically. All members of the species in a given genus of bacteria possess a DAHP-synthetase controlled by the same regulatory pattern. This explains the importance of DAHP synthetase in taxonomical studies [24]. Only one DAHP synthetase could be detected in the bacteria [13]; this enzyme is inhibited by tryptophan and to a lesser degree by phenylalanine and tyrosine.

The regulation of phenylalanine biosynthesis in *Pseudomonas aeruginosa* was studied by WALTHO [25]. Neither phenylalanine nor tyrosine affected the synthesis of chorismate mutase, prephenate dehydratase or phenylalanine transaminase. Chorismate mutase activity was inhibited by both phenylalanine and tyrosine. Prephenate dehydratase activity was inhibited by phenylalanine and stimulated by tyrosine.

Chromatography on DEAE-cellulose showed only one major peak of activity for each of the three enzymes examined.

In contrast, AHMED and CAMPBELL [26] observed in *P. aeruginosa* two chorismate mutases and two prephenate dehydratases with the same method. The activity of one chorismate mutase and one prephenate dehydratase (in-

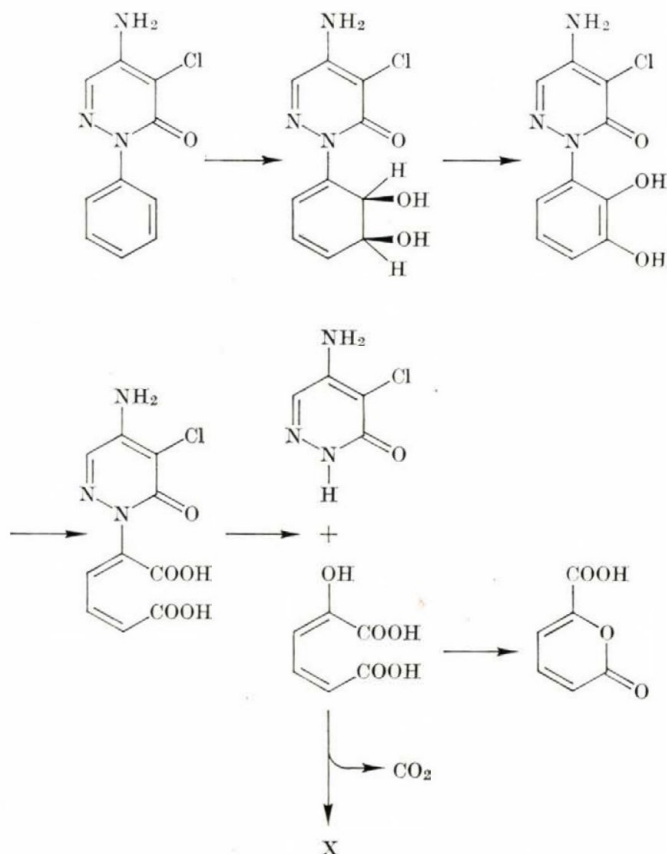


Fig. 2. Pathway for the degradation of pyrazone

hibited by phenylalanine) was found to be associated as a bifunctional enzyme. The enzymes for tyrosine biosynthesis did not appear to manifest such a physical association.

A spontaneous amber *tyr* R mutant has been isolated [27], in which constitutive synthesis of DAHP synthetase (*tyr*) and DAHP synthetase (*phe*) is suppressed by amber suppressors. This finding suggests that the *tyr* R gene product is a protein. Derepression of DAHP synthetase (*phe*) in this and in seven other spontaneous *tyr* mutants as well as in four induced *tyr* mutants provided further evidence of the involvement of the *tyr* gene product in phenylalanine biosynthesis. Evidence of the *tyr* R product being a component of the repressor rather than an enzyme involved in its synthesis or modification, has been obtained in a study of a temperature-sensitive *tyr* R mutant. This mutant is of the thermolabile type, since derepression occurs rapidly and in the presence and absence of growth.

SHIIO *et al.* [28] have made a study of the production of L-tryptophan by 5-fluorotryptophan resistant mutants of *Bacillus subtilis*. Several mutants resistant to 1500 $\mu\text{g/ml}$ of 5-fluorotryptophan produced L-tryptophan in the growing cultures. The best producer delivered 2 g/litre of L-tryptophan, when cultured in a medium containing 8% glucose without tryptophan precursors. In this mutant, anthranilate synthetase increased over 280-fold, presumably owing to a genetic depression. From this, mutants resistant to 7000 $\mu\text{g/ml}$ of 5-fluorotryptophan were derived. Among them one strain produced 4 g/litre of L-tryptophan, but also 6 g/litre of phenylalanine. The genetic alteration of this strain seemed to be involved in some metabolic regulation of the common part of the aromatic amino acid biosynthetic pathway.

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REGULATION OF TYROSINE AND PHENYLALANINE BIOSYNTHESIS IN SALMONELLA*

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Summary. Several types of 4-fluorophenylalanine resistant mutants were isolated. In one type of mutant DAHP synthetase (*tyr*) and prephenate dehydrogenase were coordinately derepressed. The mutation was linked to *aroF* and *tyrA* and was *cis*⁻ dominant by merodiploid analysis, thus confirming that it is an operator constitutive mutation (*tyrO*^c). A second type of mutation showed highly elevated levels of tyrosine pathway enzymes which were not repressed by L-tyrosine. It was unlinked to *tyrA* and *aroF*, and was *trans*-recessive in merodiploids. These properties were attributed to a mutation in a regulator gene, *tyrR* (linked to *pyrF*), that resulted in altered or non-functional aporepressor. Hence *tyrO*, *tyrA*, and *aroF* constitute an operon regulated by *tyrR*. In a third type of mutation chorismate mutase P-prephenate dehydratase was highly elevated. It was not linked to *pheA*, was located in the 95–100 min region of the *Salmonella* chromosome, and was recessive to the wild type gene in merodiploids. A mutation was, therefore, indicated in a regulatory gene, *pheR*, which specified an aporepressor for regulating *pheA*. DAHP synthetase (*phe*), specified by *aroG*, was not regulated by *pheR*, but was derepressed in one of the *tyrR* mutants, suggesting that as in *Escherichia coli* *tyrR* may regulate DAHP synthetase (*phe*) and DAHP synthetase (*tyr*) with the same aporepressor. A novel mutation in chorismate mutase is described.

Several types of 4-fluorophenylalanine resistant mutants of *Salmonella typhi-murium* were isolated. In one type of mutant, 3-deoxy-D-arabinoheptulosonic acid 7-phosphate (DAHP) synthetase (*tyr*) and prephenate dehydrogenase were coordinately derepressed, and the mutation was linked to *aroF* and *tyrA* [1]. Relevant loci are shown in Fig. 1. More recently it was shown by merodiploid analysis that this mutation was *cis*, though not *trans*, dominant (GOLLUB and SPRINSON, unpublished data), thus supporting the view that it was an operator constitutive mutation (*tyrO*^c).

A second type of 4-fluorophenylalanine resistant mutant was isolated in which tyrosine pathway enzymes were not repressed by L-tyrosine [2]. The mutants produced elevated levels of DAHP synthetase (*tyr*) and chorismate mutase T-prephenate dehydrogenase, and these enzymes as well as transaminase A were not repressed by high concentrations of tyrosine. Genetic analysis revealed that a mutation in a gene designated *tyrR* was responsible for the constitutivity of the tyrosine pathway enzymes in strains SG1, SG7, and SG9, and that *tyrR* was linked to *pyrF*. It was concluded that *tyrO*, *tyrA* and *aroF* constitute an operon regulated by *tyrR*. In strain SG1 a mutation had also

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occurred in *aroF*, the structural gene for DAHP synthetase (tyr), resulting in loss of sensitivity of this enzyme to end product inhibition. There appeared to be no relationship between loss of feedback inhibition and loss of end product repression, since derivative strains of SG1 that carried only the *tyrR* mutation behaved like the singly mutated *tyrR* strains, SG7 and SG9, in showing high constitutive levels of tyrosine specific enzymes that were not repressed by tyrosine.

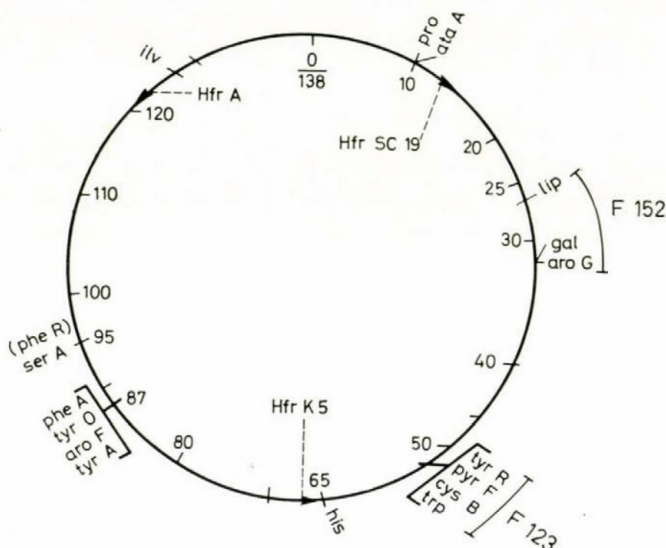


Fig. 1. Schematic linkage map of *Salmonella* and chromosomal regions carried by *E. coli* episomes

4-Fluorophenylalanine resistant mutants were also isolated in which synthesis of chorismate mutase P-prephenate dehydratase (specified by *pheA*) was highly elevated [3]. Transduction analysis showed that the mutation affecting *pheA* activity was not linked to *pheA*. Conjugation and merodiploid analysis indicated that it was in the 95- to 100-min region of the *Salmonella* chromosome, and was recessive to the wild type gene in merodiploids. A mutation was, therefore, indicated in a regulatory gene, *pheR*, which specified an aporepressor for regulating *pheA*. *pheR* mutants were found to carry a second mutation, *tyrO*. The *tyrO* mutation acted *cis* to cause increased levels of the tyrosine biosynthetic enzymes DAHP synthetase (tyr) and prephenate dehydrogenase, but it had no effect on regulation of *pheA*. DAHP synthetase (*phe*), specified by *aroG*, was not regulated by *pheR*, but was de-repressed in one of the *tyrR* mutants, suggesting that as in *Escherichia coli* *tyrR* may regulate DAHP synthetase (*phe*) and DAHP synthetase (*tyr*) with the same aporepressor [4, 5].

Chorismate mutase mutation in Salmonella. As in *E. coli*, chorismate mutase-prephenate dehydratase of *Salmonella* is a bifunctional protein controlled by the *pheA* gene, and chorismate mutase-prephenate dehydrogenase appears to be a bifunctional protein controlled by *tyrA*. However, a new type of mutant, *PC14*, was isolated from a *pheA* mutant of *Salmonella* which suggests a new interrelationship between phenylalanine and tyrosine biosynthesis (HU and SPRINSON, unpublished data; [6]). *PC14* required phenylalanine and tyrosine for growth, whereas known *pheA* and *tyrA* mutants required phenylalanine or tyrosine, respectively. Abortive and stable transduction, with phage P22, of *PC14* with various *tyrA* mutants showed the presence of groups I and II which were cotransducible with *pheA* and were different from all other *aro* mutants and from each other. Group II mutants could be further separated into two complementing subgroups, IIa and IIb, and a third noncomplementing subgroup IIc. Mutation in group I, represented by *PC14*, was a missense type which lacked chorismate mutase and had prephenate dehydrogenase. Mutants of group IIa were also missense type which had chorismate mutase and lost prephenate dehydrogenase, whereas mutations in groups IIb and IIc were all chain terminating types and were completely deficient in both enzyme activities. The group I mutants could utilize prephenate for tyrosine formation, whereas group II mutants could not. The mutation concerned with tyrosine biosynthesis in *PC14* is therefore distinct from *tyrA*, and may be designated *cmu*⁻ (lacking chorismate mutase).

Transduction with wild type cells as donor and group I as recipient gave either wild type or a phenylalanine auxotroph. Revertants of groups IIb and IIc recovered chorismate mutase and prephenate dehydrogenase, whereas revertants of *PC14* invariably required phenylalanine, and only a subsequent reversion resulted in wild type cells. Since *pheA* and *cmu* were 80% cotransducible, mutagenesis of *PC14* should have yielded *phe*⁺*cmu*⁻ and *phe*⁻*cmu*⁺. The latter genotype was recovered as phenylalanine auxotrophs (since *PC14* had prephenate dehydrogenase), whereas the former must have still required both phenylalanine and tyrosine, and was not a revertant. Hence *cmu* (the mutation in group I) controls the synthesis of both tyrosine and phenylalanine by specifying the chorismate mutase polypeptide. When it binds to the prephenate dehydrogenase, specified by *tyrA*, chorismate mutase—prephenate dehydrogenase is formed. Binding of chorismate mutase polypeptide to prephenate dehydratase, specified by *pheA*, results in formation of the chorismate mutase-prephenate dehydratase. According to this assumption each polypeptide is not active alone, but only when binding of chorismate mutase occurs to one of the other two polypeptides.

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ALLOSTERIC ENZYME-tRNA COMPLEXES AS REGULATORS OF TRANSCRIPTION OR TRANSLATION*

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Summary. The dehydrogenase activity of chorismate mutase-prephenate dehydrogenase, the allosteric enzyme of the tyrosine biosynthetic pathway in *Escherichia coli*, is inhibited by tRNA. The inhibitory effect of tRNA from *E. coli* is specific, other RNA species or polynucleotides have no inhibitory effect or only slightly influence the activity of the enzyme. While NAD only slightly influences the inhibitory effect of tRNA from *E. coli*, prephenic acid at high concentrations suppresses the inhibition. In the presence of a fixed concentration of NAD and low concentration or absence of prephenic acid the inhibitory effect of tRNA is time and temperature dependent. It seems that in the presence of tRNA and low concentration of prephenate the enzyme undergoes a time and temperature dependent conformational change. This process is reversible and can be influenced by the concentration of prephenic acid, the first precursor of the tyrosine biosynthetic pathway. The possible regulatory role of allosteric enzyme-tRNA complexes is discussed.

It was suggested by JACOB and MONOD that specific regulatory proteins, "repressors", are involved in the regulation of synthesis of inducible or repressible enzymes in the bacterial cell. According to the repressor model hypothesis, the repressor protein, structurally specified by the regulator gene, influences the rate of formation of enzymes or other proteins at the level of transcription [1]. Inducers and metabolic end-products participate as corepressors in the regulation of synthesis of enzymes by forming complexes with the specific repressor, aporepressor proteins which according to their manner of action either suppress or initiate the transcription.

During the past few years a considerable number of observations has shown that tRNA species are involved in the process of repression [2, 3]. It was found that one of the allosteric first enzymes of the aromatic amino acid biosynthesis pathway forms a specific complex with tRNA [4]. The formation of similar allosteric enzyme-tRNA complexes has been detected in the histidine and isoleucine-valine pathways too [5, 6] and it was postulated that these complexes were involved in the repression process as negative effectors [4–6]. They have been demonstrated in a few amino acid biosynthetic systems only and it is yet to be determined whether they represent typical

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or unique molecular complexes involved in the repression of amino acid biosynthetic enzyme systems.

During our studies on the allosteric enzymes and the regulation of tyrosine biosynthesis in *Escherichia coli*, we have observed that, in contrast to the simple kinetic reaction pattern obtained for tyrosine-sensitive 3-deoxy-D-arabino heptulosonate 7-phosphate synthetase in partially purified extracts [7], the same pattern for chorismate mutase-prephenate dehydrogenase is an unusually complicated one. Since we could not obtain any classical kinetic pattern with the methods used for determination of the activity of the enzyme [8, 9], it was reasonable to suppose that its activity in crude extracts or in a partially purified state is influenced not only by its substrates and allosteric effector, but also by some unidentified high molecular weight compound.

Materials and methods

Chorismate mutase-prephenate dehydrogenase was purified from *E. coli* H80cR₃ (Hfr H, thy⁻, gal⁻, aroG⁻, nad A⁻, R_{tyr}) according to KOCH *et al.* [10]. Transfer RNA was isolated from *E. coli* MRE 600 [11, 12]. Ribosomal RNA purified from *E. coli* MRE 600 [13] was kindly supplied by Dr. A. UDVARDY. Rat liver transfer RNA was isolated by the method of ROGG *et al.* [14]. *Aerobacter aerogenes* 62-1 kindly supplied by Dr. F. GIBSON was used for the preparation of chorismic acid. Prephenic acid was prepared from chorismic acid [15, 16]. Minimal medium A supplemented with the required growth factors was used [17].

Prephenate dehydrogenase activity was measured spectrophotometrically. The rate of conversion of prephenate and NAD into 4-hydroxyphenylpyruvate and NADH at 24°C in the cuvette of a Cary 15 double beam spectrophotometer was recorded continuously at 340 nm. The standard reaction mixture contained 100 μ moles Tris-HCl buffer pH 7.5, 100 μ moles potassium chloride, 2.8 μ moles prephenate, 1.0 μ mole NAD and enzyme in a final volume of 1.0 ml.

Preincubation of the enzyme with tRNA or polynucleotides was performed in incomplete reaction mixtures in a water-bath at temperatures given in the text. At the end of the preincubation period the mixtures were transferred into the cuvette of the spectrophotometer maintained at 24°C and the enzymatic reaction was initiated by completion of the reaction mixtures.

Results

The activity of partially purified preparations of chorismate mutase-prephenate dehydrogenase was influenced by pancreatic ribonuclease pretreatment. Since during the first two steps of the purification procedure [10] there is a large amount of RNA in the preparations, it was reasonable to suppose that some kind of RNA influenced the activity of the enzyme. As Fig. 1 shows, tRNA from *E. coli* MRE 600 inhibits the activity of the purified enzyme and the extent of inhibition increases with the increasing concentration of tRNA. The inhibitory effect of tRNA purified from *E. coli* is rather specific. Ribosomal RNA purified from *E. coli* MRE 600 (a mixture of 16S and 23S RNA) does not influence the activity of enzyme up to 1 mg per ml, as Table I

shows. From the point of view of specificity it was important to know whether or not the first precipitate obtained in the course of purification of tRNA from *E. coli* by isopropanol [12] affected the activity of the enzyme. After phenol extraction of *E. coli* cells, isopropanol is known to precipitate ribosomal RNA, messenger RNAs and their high molecular weight hydrolytic products, polysaccharides and lipids solubilized from the cell wall and membrane. This mixture of high molecular weight compounds does not influence the activ-

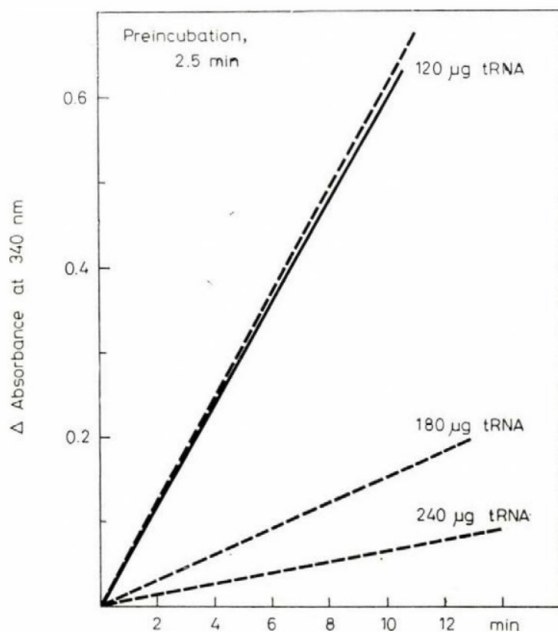


Fig. 1. Effect of tRNA on chorismate mutase-prephenate dehydrogenase activity as a function of tRNA concentration. Enzyme (31 μ g) in prephenate-free reaction mixture was preincubated at 24°C for 2.5 min in the absence of tRNA or in the presence of 120 μ g, or 180 μ g, or 240 μ g of tRNA purified from *E. coli*. At the end of the preincubation period, the reaction was initiated by the addition of prephenate, and was followed continuously at 340 nm using a Cary 15 spectrophotometer

ity of enzyme at concentrations similar to those of tRNA. At concentrations higher than 1 mg per ml it inhibits the activity of enzyme as shown in Table I. At such high concentrations the solution of "isopropanol precipitate" may inhibit the activity of the enzyme due to its tRNA content. Rat liver tRNA is less inhibitory than tRNA from *E. coli*. Synthetic polynucleotides such as poly-(A) and poly-(U) have a slight inhibitory effect only.

The inhibitory effect of tRNA on enzyme activity depends on the conditions of incubation. If the enzyme at fixed concentration was preincubated with tRNA under various conditions, different product versus time curves

were obtained. Inhibition by tRNA of enzyme activity is a time-dependent process as shown in Fig. 2. In the presence of a fixed concentration of tRNA and fixed concentration of enzyme in a prephenate-free reaction mixture, the extent of inhibition increases with time. This might indicate that the enzyme undergoes a time-dependent inhibition process that may be caused either by an equilibrium involving a slow interconversion between conformational states of the enzyme or by a change in its oligometric structure. Chorismate mutase-prephenate dehydrogenase is known to have an oligomeric structure.

Table I

Effect of RNA fractions and polynucleotides on chorismate mutase-prephenate dehydrogenase activity

Type of RNA	Concentration, μg/ml	Inhibition, per cent
tRNA from <i>E. coli</i>	120	73
rRNA from <i>E. coli</i>	500	0
	1000	0
Isopropanol precipitate from fractionation of <i>E. coli</i> tRNA	1000	3
	2000	21
	4000	54
Rat liver tRNA	200	25
	1000	47
poly-(U)	500	20
poly-(A)	1000	26

31 μg enzyme in prephenate-free reaction mixture was preincubated at 24°C for 5 min in the presence or absence of the RNA fraction or polynucleotide listed in the Table. At the end of the preincubation period the samples were transferred into the cuvette of a Cary 15 spectrophotometer maintained at 24°C and the reaction was started by addition of prephenate to the reaction mixture

At low ionic strength and in the absence of substrates, the oligomer enzyme has two subunits [18]. It is yet to be determined whether the substrates, allosteric effectors or tRNA, are influencing the conformation and oligomeric structure of the enzyme. Inhibition of activity being a time-dependent process, it seems highly probable that the catalytically active conformation of the enzyme is different from the tRNA inhibited form.

The effect of temperature on the inhibitory effect of tRNA is shown in Table II. At a fixed concentration of tRNA, the degree of inhibition increases with the increase in temperature. This may suggest that the conformation or aggregation states of the enzyme are affected by the temperature.

In the experiments described previously the enzyme was incubated with tRNA in the absence of prephenic acid. It was interesting to study the effect of different concentrations of prephenic acid or NAD on the inhibition by tRNA.

Table II

Effect of temperature on the inhibitory effect of purified E. coli tRNA on chorismate mutase-prephenate dehydrogenase activity

Preincubation temperature	Inhibition, per cent
0°C	36
24°C	73
37°C	95

31 μg enzyme in prephenate-free reaction mixture was preincubated with 120 μg tRNA from *E. coli* at different temperatures for 5 minutes. At the end of the preincubation period the sample was transferred into the cuvette of the Cary 15 spectrophotometer maintained at 24°C and the reaction was initiated by the addition of prephenate. Control sample without tRNA was incubated under the same experimental conditions. The inhibition is expressed as the difference in the reaction rate at 24°C after preincubation of the enzyme with or without tRNA at the given temperature

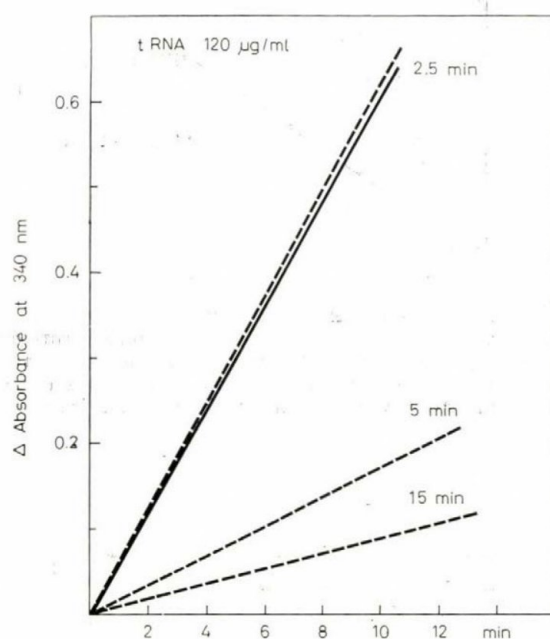


Fig. 2. Inhibitory effect of tRNA from *E. coli* on chorismate mutase-prephenate dehydrogenase activity as a function of preincubation time. Enzyme (31 μg) in prephenate-free reaction mixture was incubated at 24°C in the absence or presence of 120 μg tRNA for 2.5 min, for 5 min or for 15 min. At the end of the preincubation period, the catalytic reaction was started by the addition of prephenate. The reaction was followed as in Fig. 1

As Fig. 3 shows, the inhibitory effect of a fixed concentration of tRNA depends on the composition of the preincubation mixture. If the enzyme is incubated with tRNA in buffer in the absence of substrates and the catalytic reaction is initiated by the addition of both substrates, the inhibition will be of maximum intensity. The addition of NAD to the preincubation mixture will affect the inhibition only slightly, but if the enzyme is incubated with tRNA in buffer in the presence of prephenic acid and the catalytic reaction

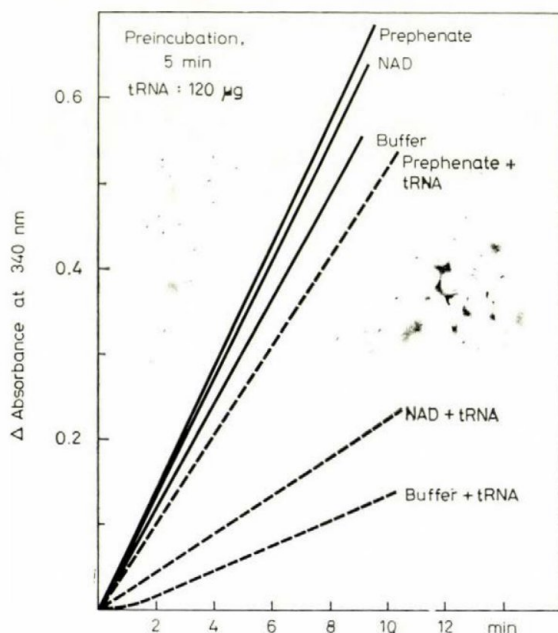


Fig. 3. Inhibitory effect of *E. coli* tRNA on chorismate mutase-prephenate dehydrogenase activity in the presence of different components of the reaction mixture during the preincubation period. Enzyme (31 μ g) was preincubated at 24°C for 5 min in the presence of buffer or NAD or prephenate without tRNA or with 120 μ g tRNA. At the end of the preincubation period, the reaction was initiated by completing the reaction mixture and was followed as in Fig. 1

is initiated by the addition of NAD, the inhibitory effect will be quite weak. It seems from the experiments shown in Fig. 3 that prephenic acid — a specific metabolite of the aromatic amino acid biosynthetic pathway — exerts in this respect a stronger influence than does NAD, a coenzyme of many dehydrogenases. As Fig. 4 shows, if the enzyme is incubated in buffer with a fixed concentration of tRNA in the presence of a fixed concentration of NAD and the reaction is started by the addition of increasing concentrations of prephenate, the inhibitory effect of tRNA decreases with the increasing prephenate concentration. Thus, there is an apparent competition between

tRNA and prephenic acid. Since the two compounds are different in structure, the observed competition may reflect a shift in the equilibrium between the substrate or tRNA binding conformation of the enzyme. As Fig. 4 shows the inhibition of enzyme activity by tRNA seems to be a reversible process.

In the above experiments the natural mixture of stripped tRNA purified from *E. coli* MRE 600 was used, but it is supposed that only a fraction of the mixture is active in the inhibition of chorismate mutase-prephenate dehydro-

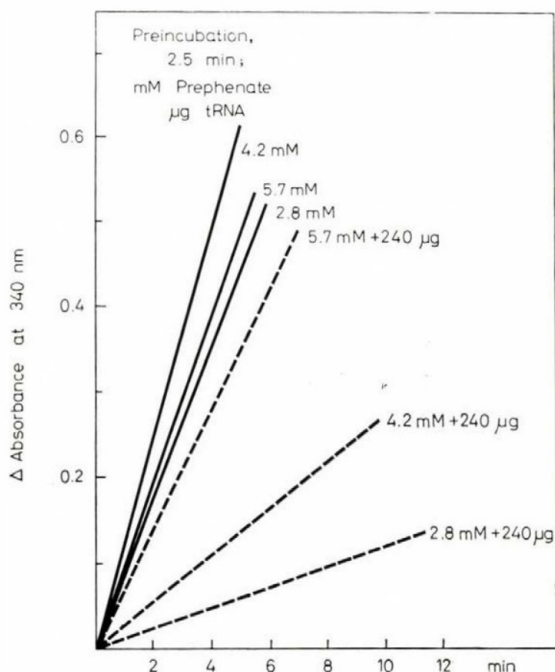


Fig. 4. Suppression by prephenic acid of the inhibitory effect of tRNA on chorismate mutase—prephenate dehydrogenase activity. Enzyme (40 μ g) was preincubated at 24°C for 2.5 min in buffer containing NAD without tRNA or with 240 μ g tRNA. The catalytic reaction was started by the addition of 2.8 mM or 4.2 mM or 5.7 mM prephenic acid

genase. Using purified stripped tRNA charged with [14 C] tyrosine in the preincubation mixture, the inhibitory effect increases as it is seen in Fig. 5, and it is highly probable that tyrosyl-tRNA is the fraction responsible for the inhibition of chorismate mutase-prephenate dehydrogenase.

Discussion

The physiological role of the allosteric enzyme-tRNA complexes is unclear. The corepressors in the repression process controlling the synthesis of enzymes of the histidine and isoleucine-valine biosynthetic pathways are not

their free amino acid end-products but the charged aminoacyl-tRNAs [2, 3]. The allosteric enzymes of some amino acid biosynthetic pathways are able to form specific complexes with the stripped or charged tRNA *in vitro* [4-6, 19, 20]. The allosteric enzyme of the histidine biosynthetic pathway is involved in the repression process [21-23] similarly as threonine deaminase, the allosteric enzyme of the isoleucine-valine pathway, plays a role in the regulation of enzymes participating in the biosynthesis of isoleucine and valine [6, 24-26].

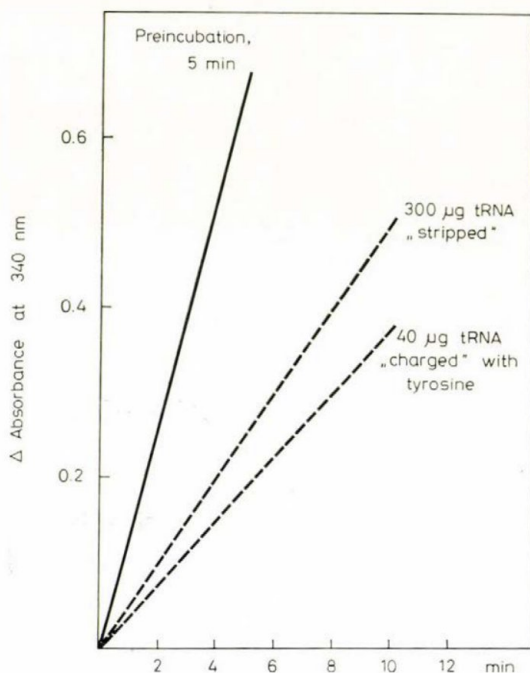


Fig. 5. Inhibition of chorismate mutase-prephenate dehydrogenase activity by "stripped" tRNA or with tRNA charged with tyrosine. Enzyme (42 μ g) in prephenate-free reaction mixture was preincubated at 24°C for 5 min in the absence of tRNA or in the presence of 300 μ g stripped tRNA or in the presence of 40 μ g tRNA charged with tyrosine. At the end of the preincubation period the reaction was initiated by the addition of NAD and was followed continuously at 340 nm

Thus, the mechanism of repression may in some cases be more complicated than the model proposed by JACOB and MONOD [1].

The allosteric first enzymes of certain amino acid biosynthetic pathways appear to be involved in the regulation of the rate of enzyme synthesis as well as the rate of formation of the amino acid end-product. According to this hypothesis, some allosteric enzymes have a dual role in the regulation process. In view of the failure to find a "true" regulator gene for the histidine biosynthetic pathway, it was supposed that the allosteric enzyme of the system

encoded by its structural gene could be the aporepressor [27]. Studies on the regulation of the isoleucine-valine pathway and the tRNA binding properties of threonine deaminase suggest that the immature form of the allosteric enzyme may be the aporepressor [6]. According to this theory, the allosteric enzymes are negative regulatory elements, aporepressors, in both the histidine and the isoleucine-valine biosynthetic systems. It remains to be established whether the allosteric enzymes as negative regulatory elements are in fact aporepressors or, else, components of some unclarified regulatory complexes. This question has support from studies on repressible systems. Histidyl-tRNA synthetase seems to represent a positive regulatory element in the histidine biosynthetic system [28, 29]. Deletion of the structural gene of threonine deaminase does not affect the regulation of the isoleucine-valine biosynthetic enzyme [30]. Since threonine deaminase is certainly involved in the regulation of the isoleucine-valine pathway, the immature form of the allosteric enzyme may be a component of a regulatory complex. In view of the complexity and number of the elements involved in the repression process of biosynthetic systems, these regulatory complexes or some of their components might regulate both the rate of transcription and the rate of translation.

It seems then that a simple substitution of the allosteric enzyme encoded by its structural gene for the aporepressor encoded by the regulator gene is not sufficient to explain the results obtained during the last few years. In view of the apparent complexity of the repression process in the biosynthetic system the basic idea of a negative regulation as proposed by JACOB and MONOD is probably valid, but the components and structure of the repressor complex may vary considerably.

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MACROPHAGE MIGRATION INHIBITION AS AN IN VITRO CORRELATE OF CUTANEOUS DELAYED SKIN REACTION

III. RELATIONSHIP WITH SPECIFIC RESISTANCE

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Summary. The eventual correlation between delayed skin reaction, macrophage migration inhibition and specific resistance has been investigated. No correlation between delayed skin reaction and specific resistance has been established but a positive correlation between macrophage migration inhibition and specific resistance may be suggested.

It has been shown [1] that macrophage migration inhibition (MMI) and delayed skin reaction (SR) are correlated only in the case of a positive SR. A negative SR is associated with a positive MMI test in 2/3 of cases. The increase of the applied intracutaneous PPD dose from 10 U to 100 U did not alter the results. Though MMI had many advantages over the SR [2–4], the question remained open whether the MMI was more sensitive or less specific than the SR.

Our present investigations had the aim to clarify this question.

Materials and methods

Experiments were performed in 100 guinea pigs; 72 yielded evaluable data. The animals were divided into three groups: (1) 30 guinea pigs for virulent tuberculosis infection; (2) 30 guinea pigs for BCG vaccination followed by virulent tuberculosis infection; (3) 40 guinea pigs were BCG vaccinated and used for MMI test.

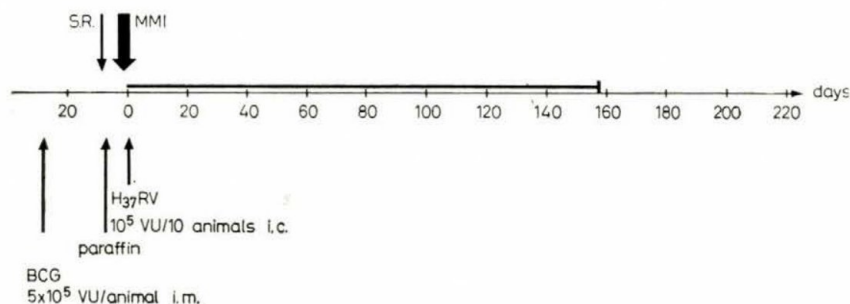


Fig. 1. Experimental design

Fig. 1 shows the experimental design. After an observation period of 6 weeks the guinea pigs of groups 2 and 3 were vaccinated intracutaneously with 5×10^5 live BCG bacteria/animal. All animals in the three groups were skin-tested after 21 days with 10 U and 100 U of PPD (a gift of Statens Seruminstitut, Copenhagen) and read on the 23rd postvaccination day. The 3rd group received 25 ml paraffin oil intraperitoneally on the same day. These animals were used for MMI test carried out by the method of GEORGE and VAUGHAM [5] as modified by us [6] on the 27th, 28th and 29th days after vaccination. MMI was carried out with 15 μ g/ml PPD from the same sample as used for skin testing. The guinea pigs of groups 1 and 2 were infected with virulent H₃₇RV *M. tuberculosis* bacteria on the 28th day after vaccination. According to our experimental plan, skin tests and MMI show the cellular immune responsiveness of our animals at the time of infection with virulent bacteria. Mean survival in groups 1 and 2 was calculated on the 205th day after tuberculosis infection.

Results

Fig. 2 shows the correlation between MMI test and SR of the same animals. Evaluation was made as follows. MMI: + inhibition more than 2 SD; $\pm$ inhibition between 1 and 2 SD; — inhibition less than 1 SD. SR: + induration after 10 U PPD more than 2 mm; $\pm$ induration after 100 U PPD more than 2 mm; — no induration.

MMI \ SR	+	$\pm$	—	Σ
+	3	0	0	3
$\pm$	11	4	0	15
—	2	0	0	2
Σ	16	4	0	20

Fig. 2. Correlation between MMI and SR

The findings agreed with our earlier results in that in the case of SR positivity the MMI was also positive. Out of 15 SR $\pm$ cases MMI was positive in 11 and $\pm$ in 4 cases. In 2 SR negative cases MMI was positive.

Fig. 3 demonstrates the survival curve of the guinea pigs in groups 1 and 2. There were essential differences (i) in the slope of survival curve, and (ii) mean survival time which was 79 days for the infected (control) animals and more than 141 days for the BCG vaccinated guinea pigs infected with tuberculosis. Five animals were still alive when the results were evaluated.

The skin responses are marked on the survival curves. SR positive animals died early after tuberculosis infection; only a single one survived.

The same was the case for the $SR \pm$ animals; in this group 3 animals survived. The lack of a correlation between SR and specific resistance was obvious. Most of the SR negative animals had, however, a positive MMI test and it was supposed that they were protected from virulent tuberculosis. Still only 1 SR negative animal died within the mean survival time of tuberculosis infected animals, the others had some though not sufficient protection.

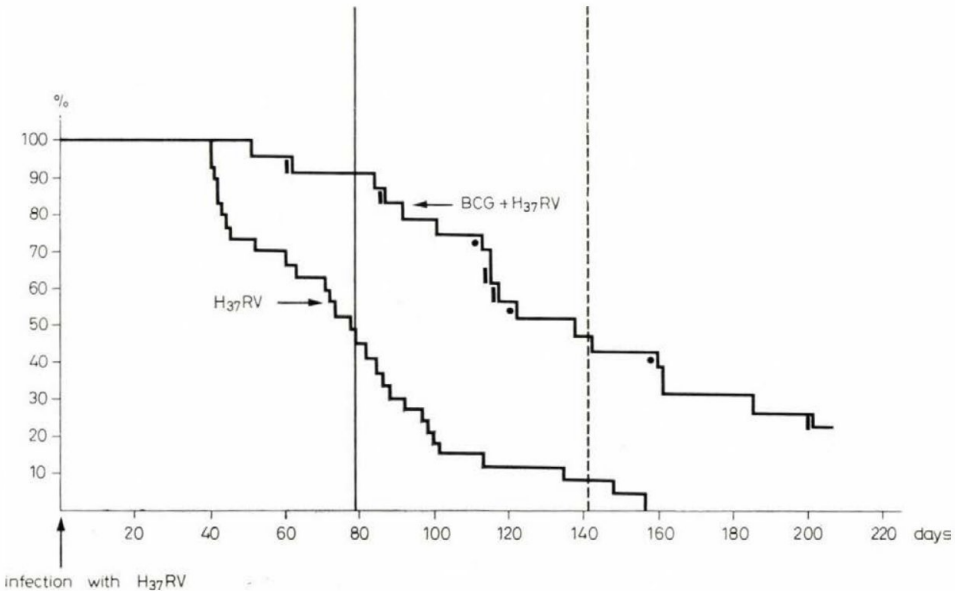


Fig. 3. Survival of guinea pigs infected with $H_{37}RV$ and with $BCG + H_{37}RV$. Mean survival time, 79 days for $H_{37}RV$ -infected animals (solid line) and 141 days for $BCG + H_{37}RV$ -infected animals (broken line). Skin reaction of surviving $BCG + H_{37}RV$ -infected animals: 1 positive, 3 weakly positive and 1 negative

Discussion

It is difficult to establish a correlation between MMI and specific resistance, since MMI and specific resistance cannot be investigated in the same animal. It was certain that both the MMI and the survival time of BCG vaccinated guinea pigs pointed to a good protection, but we could speak of a close correlation only if all the SR positive and $SR \pm$ animals would have survived and so would have 2/3 of the SR negative ones. This was obviously not the case. In spite of this, the correlation seemed to be better between specific resistance and MMI than between specific resistance and SR, because in the latter case there was no correlation at all.

The question arises whether a "close correlation" could exist between a test *in vitro* and one *in vivo*. The MMI depends on MIF production which

is in close connection with lymphocyte activation. Lymphocyte activation plays an essential role also in the development of specific resistance but is obviously not the sole determining factor. Specific resistance is the response of the whole living animal. It is influenced by several known factors (food intake, temperature, metabolism, endocrine functions) and also by unknown ones. This is suggested by the investigations of MAKINODAN and PETERSON [7] who showed that the diminished lymphocyte response characteristic of old age depends on lymphocyte function in 90% and on the environment, the aged body of the animal, in 10%.

It has also to be considered that MMI and SR show the animal's immune response at the time of infection with virulent tuberculosis bacteria. This infection, however, means a second sensitization for the animal which may improve immunity in certain cases, but may diminish it if the infection occurs before the immune response had developed. This possibility has been suggested by the fact that only 1 out of 9 SR negative animals survived, though 6 should have survived according to our earlier experiments.

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IMMUNOCHEMICAL EXAMINATION OF SOLUBLE ANTIGENS IN THE SERUM OF Balb/c MICE INFECTED WITH RAUSCHER LEUKAEMIA VIRUS

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Summary. The serum of Balb/c mice infected with Rauscher leukaemia virus contained soluble antigens characterized by α_2 and β globulin electrophoretic mobility; their respective molecular weights, as determined with gel filtration, were 40 000 and 120 000. The antigens differed in specificity and their corresponding determinants were present on the surface of leukaemic cells. For Balb/c mice both antigens, for DBA/1 and C57B1/10Sn mice only the antigen showing α_2 mobility was immunogenic.

The serum of rats with sarcoma induced by carcinogenic substance [1, 2] and of mice with Friend, Rauscher, Moloney [3], Gross [4] and Graffi [5] leukaemia contains antigens not bound to cells or virions. The relationship of these soluble antigens to surface factors of the tumour cell was studied in detail in mice infected with Gross and Friend leukaemia. By the use of isologous and heterologous immune serum, AOKI *et al.* [6] observed six kinds of specificity in Gross soluble antigens. None of these antigens was immunogenic in AKR and C58 mouse strains, members of which produced readily Gross soluble antigens. Whether the six kinds of antigens are bound to one or to more molecules, has not yet been elucidated; type-specific determinants of the envelope of leukaemia viruses and the surface antigens induced by them are present probably on one molecule or on one molecule complex [7, 8]. In Balb/c mice with Friend leukaemia LILLY and NATHENSON [9] detected two soluble antigens different in molecular weight. Immune sera prepared in mice of the same strain inhibited the cytotoxic effect of both antigens and, accordingly, the antigen determinants were assumed to be identical. The soluble Rauscher antigen [3] has not been investigated in detail.

In view of the above findings, it seemed of interest to determine the number of the components of soluble Rauscher antigen and their relationship to one another and to the surface antigens of the tumour cells. The present work deals with antigens immunogenic to mice.

Materials and methods

Virus. The Rauscher leukaemia virus was obtained from Dr. V. M. Bergolts (Gertsen Institute for Oncology, Moscow) and was maintained in Balb/c mice. The experimental animals were infected intraperitoneally with 0.2 ml cell-free supernatant of the 20% spleen-homogenate of Rauscher leukaemic Balb/c mice.

Animals. Stock inbred Balb/c, DBA/1 and C57B1/10Sn mice obtained from the Central Research Institute of the National Blood Service, Budapest, were bred in our institute and the offsprings were used in the experiments.

Soluble antigens. Eight-week-old Balb/c mice were infected with Rauscher virus and bled 18–20 days later. The virus was removed by passing the serum through 50 nm membrane filters (SM 11310, Sartorius Membranfilter GmbH, Göttingen).

Immune sera. The soluble antigens were detected with sera of Balb/c, DBA/1 and C57B1/10Sn mice immunized with formalinized vaccine and by the use of the serum of DBA/1 and C57B1/10Sn mice infected with Rauscher virus. Formalinized vaccine was prepared from the spleen of leukaemic Balb/c mice and was used for immunization as described by FINK and RAUSCHER [10]. The first injection was given with or without complete Freund adjuvant (Difco, Detroit), whereas the booster dose contained no adjuvant. DBA/1 and C57B1/10Sn mice reacting to Rauscher virus infection with a significant antibody response [11] were bled 22 days after injection of the virus. The immune sera obtained from DBA/1 and C57B1/10Sn mice were absorbed with spleen cells from normal Balb/c mice [12]. Rabbit anti-mouse serum was prepared in the Institute for Serobacteriological Production and Research Human, Budapest.

Immunoelectrophoresis. SCHEIDEGGER's micromethod [13] was used. Slides were overlaid with 1.5% agar (Noble Special, Difco, Detroit) dissolved in boric acid-tetraborate buffer (0.05 M, pH 8.6). Electrophoresis was performed at a gradient of 4 V/cm for 4 hr.

Block electrophoresis was carried out on 160 × 80 mm glass plates covered with a 5 mm thick layer of 1.5% agar dissolved in tris-boric acid-EDTA buffer (0.1 M pH 8.6). After cutting a 60 × 2 mm trench and filling with serum, electrophoresis was performed at 400 V and 4°C for 16 hr. Migration of proteins was checked with bromophenol blue-stained albumin. Then a filter paper strip immersed in buffer was placed on the surface of the agar layer and stained with amido-black (Serva, Heidelberg) to show the position of the serum protein fractions. The agar block was then cut into strips and the protein fractions were extracted with saline and concentrated to the original volume on coarse Sephadex G-25 (Pharmacia, Uppsala). Subsequently, the protein fractions were dialysed against saline at 4°C overnight.

Immunodiffusion. Double diffusion was performed as described by OUCHTERLONY [14] in 1.5% agar prepared with boric acid-tetraborate buffer (0.05 M, pH 8.6).

For gel filtration, 80 × 2.6 cm columns of Sephadex G-200 prepared with Sörensen phosphate buffer (0.05 M, pH 7.3) were used; V_0 was determined with blue dextran 2000 (Pharmacia, Uppsala). Globulin fractions α_2 and β isolated from the virus-free mouse serum by means of block electrophoresis and concentrated to the original volume were run and after concentration the eluant fractions were examined with immunodiffusion for the presence of soluble antigens. Molecular weight was calculated on the basis of ANDREWS' correlation [15].

Indirect immunofluorescence inhibition test. From an immune serum prepared in Balb/c mice serial twofold dilutions (1 : 2–1 : 64) were made in virus-free leukaemic serum, its α_2 and β globulin fractions and normal Balb/c serum. The tubes were incubated at room temperature for 30 min, then 0.05 ml samples were taken for antibody titration. The antibody content was determined by indirect membrane immunofluorescence using spleen cells of leukaemic Balb/c mice. In the first stage of the reaction, 0.05 ml serum dilution was added to 2×10^6 spleen cells, the suspension was incubated at 37°C for 20 min, then washed three times with PBS. In the second stage, 0.05 ml fluorescein isothiocyanate-labelled swine anti-mouse globulin (Sevac, Prague) was added to the cells and after 20 min incubation at 37°C the cells were washed three times. Finally, the cells were resuspended in one drop of PBS, dried on slides and examined by fluorescence microscopy. The index was calculated as described by KLEIN and KLEIN [16].

Results

Detection of soluble Rauscher antigens by immunoelectrophoresis. Immune sera prepared in Balb/c mice with or without Freund adjuvant showed the presence in virus-free leukaemic serum of two antigens different in electrophoretic mobility. The antigens were specific for Rauscher leukaemia, as they were absent from normal Balb/c serum. Parallel application of anti-Rauscher immune serum and rabbit anti-mouse serum allowed to demonstrate that the antigens corresponded in mobility to the α_2 and β globulin fractions (Fig. 1).

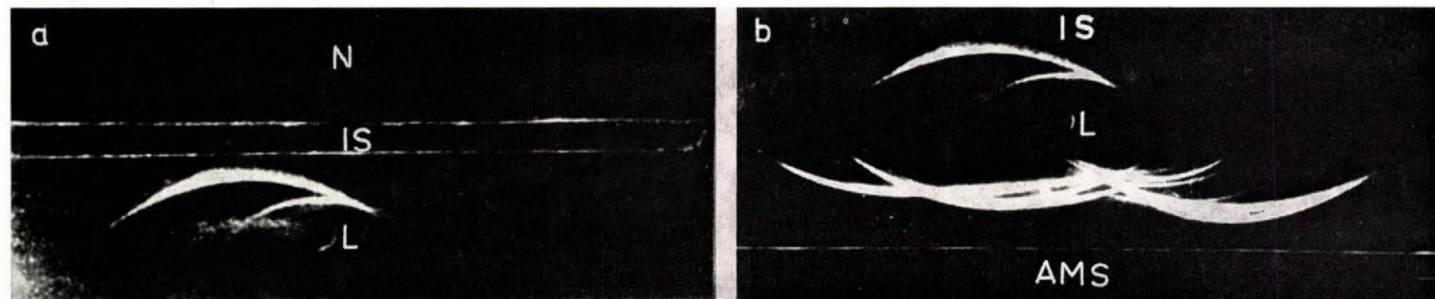


Fig. 1. Detection of soluble tumour-specific antigens in the serum of Rauscher leukaemic Balb/c mice. The immune serum was prepared in Balb/c mice. (a) N = normal serum, IS = immune serum, L = leukaemic serum; (b) IS = immune serum, L = leukaemic serum, AMS = anti-mouse serum

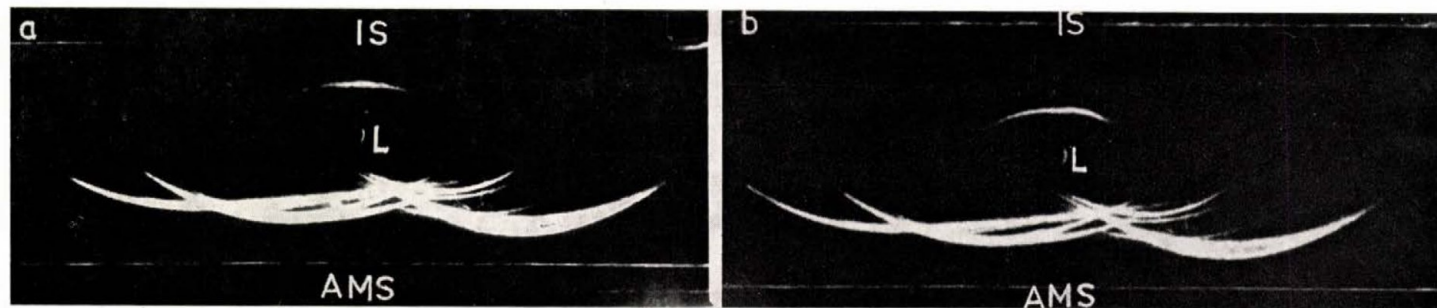


Fig. 2. Detection of antigen of α_2 -type electrophoretic mobility in the serum of Balb/c mice infected with Rauscher leukaemia. The immune sera were prepared in DBA/1 and C57B1/10Sn mice. (a) IS = DBA/1 immune serum, L = leukaemic serum, AMS = anti-mouse serum; (b) IS = C57B1/10Sn immune serum, L = leukaemic serum, AMS = anti-mouse serum

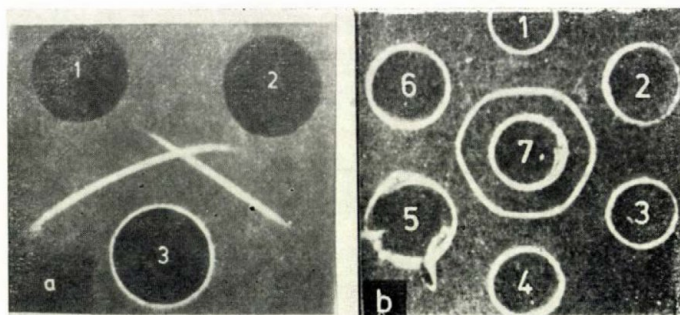


Fig. 3. Immunodiffusion experiments with soluble tumour-specific antigens and immune sera reacting with α_2 -type antigen. (a) 1 = α_2 globulin fraction, 2 = β globulin fraction, 3 = Balb/c immune serum; (b) 1 = Balb/c immune serum, 2 = DBA/1 immune serum, 3 = C57B1/10Sn immune serum, 4 = Balb/c immune serum, 5 = DBA/1 immune serum, 6 = C57B1/10Sn immune serum, 7 = α_2 globulin fraction

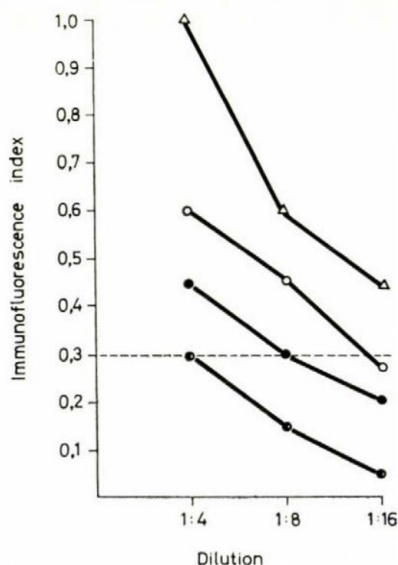


Fig. 4. Blocking effect of soluble antigens on tumour-specific antibodies. Immunofluorescence inhibition test. Δ control; $\circ$ α_2 globulin fraction; $\bullet$ β globulin fraction; $\bullet$ whole leukaemic serum

As seen in Fig. 2, in DBA/1 and 357B1/10Sn mice only the α_2 -type antigen was immunogenic, independently of the use of formalinized virus + Freund adjuvant, simple formalinized vaccine or live Rauscher virus.

Identity and specificity of soluble antigens. From virus-free leukaemic serum the α_2 and β globulin fractions were isolated by agar block electrophoresis. In double gel diffusion test the antigens showed crossing lines and were, therefore, of different specificity (Fig. 3a). In the α_2 globulin fraction of leu-

kaemic serum the same antigen was detected with immune sera prepared in Balb/c, DBA/1 and C57B1/10Sn mice (Fig. 3b).

The α_2 and β globulins isolated from leukaemic serum by agar block electrophoresis were gel-filtered, the fractions were concentrated and examined for the presence of soluble antigens with Balb/c immune serum. In this manner the molecular weight of the α_2 -type antigen was estimated at 40 000, that of the β -type antigen at 120 000.

The relationship between the soluble antigens and surface antigens of Rauscher leukaemia cells was examined with the membrane immunofluorescence inhibition test. Virus-free leukaemic serum and its α_2 and β fractions decreased the activity of the immune serum more markedly than did the control (normal) serum (Fig. 4). This finding indicates that antigen determinants corresponding to the soluble antigens are present on the surface of the tumour cell, in other words, the soluble antigens are of membrane origin.

Discussion

The serum of mice infected with Rauscher leukaemia virus contains two soluble antigens different in molecular weight, electrophoretic mobility and specificity. The presence of unbound antigen in serum is due to the fact that the Rauscher virus causes a definite immunosuppression [18] and thus immune complexes fail to develop. The finding that cell surface antigens are immunogenic even in the most susceptible mouse strain, is in agreement with the results of LILLY and NATHENSON [9] for Friend soluble antigens. In contrast, AOKI *et al.* [6] have shown that Gross-virus-induced membrane antigen determinants in the serum of AKR and G58 mice react only with immune sera prepared in other mouse strains or in rats. The difference may be explained by the fact that vertical transmission of infective Friend or Rauscher virus, unlike of Gross virus, cannot be accomplished even in the most susceptible mouse strains [19]. According to LILLY and NATHENSON [9], as assumed on the basis of the cytotoxicity-inhibiting power of the serum, Friend soluble antigens different in molecular weight are probably identical in specificity. The indirect immunofluorescence inhibition test showed that both kinds of Rauscher soluble antigens blocked the antibodies bound to the surface of the leukaemia cell. It is known that the Friend and Rauscher agents belong to the same serotype of murine leukaemia viruses and are responsible for the same disease [20]. Chromatographic studies showed that the soluble antigens of the two viruses were similar in molecular weight [9]. Accordingly, two soluble antigens of different specificity probably appear not only after Rauscher virus infection, but also in the serum of Balb/c mice infected with Friend virus adapted to this strain of mice [21]. It is interesting that for DBA/1 and

C57B1/10Sn mice only the α_2 -type antigen is immunogenic. As the relative resistance against Rauscher virus of DBA/1 mice is associated with a tumour-specific immune response [18], the appearance in the serum of the antigen may influence the course of the disease by decreasing the effectiveness of cytotoxic lymphocytes and antibodies. Immunochemical characterization of the antigen offers a means for the detection of its immune complexes by gel-filtration. Further experiments are needed to show whether Rauscher soluble antigens are virion or virus-induced surface factors.

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A FIVE-YEAR SURVEY OF HUMAN *YERSINIA* ENTEROCOLITICA INFECTIONS IN HUNGARY

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Summary. (i) Human infections associated with *Yersinia enterocolitica* in Hungary in the years 1969-1974 have been surveyed. During this period the public health laboratory network isolated 1355 strains from 1096 persons. The number of isolates according to sero-groups was: 1343 O3, 6 O9, 2 O1, 2a, 3 and 1 strain for O5, O6, O10 and O15 each. (ii) A total of 2192 serum specimens from patients gave positive agglutination reaction with antigen O3 in 26.8%, with antigen O9 in 3.2%. (iii) Fifty-six per cent of bacteriologically positive persons had enteritis. Other clinical forms (pseudoappendicitis and other abdominal complaints, erythema nodosum, rheumatoid arthritis) were encountered in 0.1-2.7%. Symptomless excretors of *Y. enterocolitica* amounted to 23.1% of all positive persons. (iv) Patients with enteritis and symptomless excretors were rather evenly distributed between 10 and 60 years of age; 1-9-year-old children were affected more frequently (47.7% of all positive persons). Sex distribution was, males : females = 1.5 : 1. In seasonal incidence yersiniosis differed from other enteric diseases: it showed a peak in the autumn-winter months. Sporadic cases and family outbreaks were the most frequent; epidemic infections in nurseries were also recorded. (v) Out of 59 animal strains 39 group O3 cultures were isolated from pigs, which may be assumed as the most important reservoir of yersiniosis in Hungary.

In the sixties, papers from a number of European countries [1-10] dealt with the incidence of "Pasteurella X" named later *Yersinia enterocolitica* upon the proposal of FREDERIKSEN [11]. These reports gave a considerable impulse to the research of this organism, the basis of which had been set about 20 years earlier in the U.S.A. by SCHLEIFSTEIN and COLEMAN [12] and GILBERT [13]. Advance in the study of the agent was summarized in symposia held in Paris in 1967 [14] and in Malmö in 1972 [15]. These observations have shown that yersiniosis occurs not only in Europe and in North America [16, 17] but also in Africa [18] and Asia [19].

Systematic studies on yersiniosis in Hungary were started in 1969 [20, 21]. The first isolation of *Y. enterocolitica* in this country was made by RÉDEY in 1965. His strain was identified in 1969 in this institute [22]. From the spring of 1970 onward, *Y. enterocolitica* strains were isolated in increasing numbers all over the country. By the end of 1974, the agent had been found in all but 3 counties of Hungary.

In the present paper we give an account of yersinia isolations during a five-year period.

Materials and methods

Isolation of strains. The cultures examined were isolated partly in this laboratory (covering the Pest county region), partly in public health laboratories in different areas of the country. Faecal specimens were streaked on 2 plates of desoxycholate citrate (DC) agar. Samples received after appendectomy were swabbed with cotton wool and seeded on 2 DC plates. One of the plates was incubated at 37°C for 18–20 hr, the other at 22–25°C, then both were left to stand at room temperature for 24 hr. For enrichment a few drops of faecal suspension were inoculated into Wauters' medium [23], incubated at room temperature for 4 days and subcultured onto DC plates after 2 and 4 days. Lymph nodes were dissected into small pieces, homogenized in saline and seeded on DC and blood agar. Blood specimens were cultured as usually. Suspicious colonies seen on the primary plates were transferred to triple-sugar-thiosulphate-iron medium on which *Y. enterocolitica* produced acid without gas in the butt and in the slant.

Biochemical reactions were performed from blood agar plates or from nutrient agar slants. The following characters were routinely determined: motility and Voges-Proskauer reaction at room temperature and at 37°C, oxidase, catalase, urease, ONPG, ornithine decarboxylase, tryptophan deaminase, acid production in adonitol, mannitol, lactose, xylose and glucose.

Serological examination of the isolates was performed from 48-hr DC and blood agar plates (incubated at room temperature for at least 24 hr) with slide agglutination in O sera.

Agglutination test with patients' sera and its evaluation were done as described earlier [24].

Antibiotic sensitivity testing was carried out with Resistest discs (Institute for Sero-bacteriological Production and Research Human, Budapest) as standardized for the Hungarian public health laboratory network [25].

Results

Incidence of *Y. enterocolitica*. Table I shows that there was a considerable yearly increase in the number of positive persons and isolates. Fig. 1 presents the geographic division of the country with the year of the first isolation of

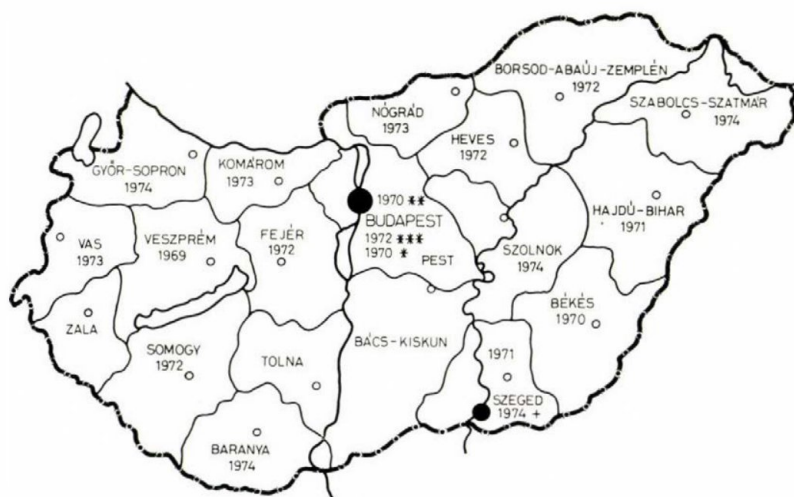


Fig. 1. Regional distribution of *Y. enterocolitica* isolations from humans in Hungary, 1969–1974. Each figure in the map means the year of the first isolation of the agent in the corresponding area, with the exception of Veszprém where the year of identification of the first isolate is shown. * National Institute of Public Health, Budapest; ** Municipal Public Health Station, Budapest; *** László Central Infectious Disease Hospital, Budapest; + Public Health Station, Szeged

Y. enterocolitica in each area. By the end of 1974, only 3 regional laboratories failed to isolate the agent. (After submission of this paper the number of "white spots" decreased to two counties.)

Table I

Incidence of Y. enterocolitica isolates in humans in Hungary, 1969-1974

Years	No. of positive persons	No. of strains
1969	1	1
1970	18	42
1971	40	59
1972	246	324
1973	345	438
1974	446	491
Total	1096	1355

Table II summarizes the geographic distribution of *Y. enterocolitica* isolations in the years 1969-1974. The great variety in the number of isolates may be attributed to differences in the incidence of outbreaks and in the ability of the laboratories to recognize the agent, rather than to the number of faecal specimens examined. In this respect it may be noted that in 1973, owing to an outbreak with 80% positive results, the total number of our isolates (107) was almost the same as that of the Budapest Public Health Station (109) although the latter examined about three times more faecal specimens than we did.

Table III shows the serogroup distribution of *Y. enterocolitica* according to laboratories. The overwhelming majority of O3 strains is in agreement with their predominance in other European countries and Canada [26-30].

Antibody response in patients. Table IV shows antibody titres against *Y. enterocolitica* antigens O3 and O9 in a total of 2192 sera taken from patients. The antibody response was regarded as negative at titres 1 : 10-1 : 20, doubtful at 1 : 40, and positive at 1 : 80 or above. In the 5-year period, with antigen O3, 1509 (68.8%) negative, 96 (4.4%) doubtful and 587 (26.8%) positive; with antigen O9, 2096 (95.7%) negative, 25 (1.1%) doubtful and 71 (3.2%) positive results were obtained. From 1971 onward, sera reacting with antigen O9 were absorbed with *Brucella*, then retested to avoid false reactions due to an antigenic relationship between the two antigens [31-37].

Clinical forms of yersiniosis. The majority of *Y. enterocolitica* isolations was made from cases of enteritis (619 patients, 56.5% of all positive persons).

Table II

Number and percentage of *Y. enterocolitica* isolations from humans in Hungary, 1969-1974

Public health laboratories	No. of positive persons	Strains	
		Number	Per cent
Baranya	1	1	0.1
Békés	44	46	3.4
Borsod-Abaúj-Zemplén	45	45	3.3
Csongrád	8	8	0.6
Fejér	27	29	2.1
Győr-Sopron	5	5	0.4
Hajdu-Bihar	7	7	0.5
Heves	37	40	3.0
Komárom	4	4	0.3
Nógrád	7	8	0.6
N.I.P.H.*	152	197	14.5
Somogy	14	14	1.0
Szabolcs-Szatmár	1	1	0.1
Szolnok	4	4	0.3
Vas	19	20	1.5
Veszprém	285	430	31.7
Budapest	393	446	32.9
László Hospital**	33	36	2.7
Szeged	10	14	1.0
Total	1096	1355	100.0

* National Institute of Public Health, Budapest

** Budapest

The enteritis was accompanied by pyrexia in 183 patients. In a few instances the temperature rose to 40°C; in 50 patients the enteric symptoms were preceded or accompanied by bronchitis, grippe or sore throat. Gastritis was diagnosed in 12 patients with enteritis. Symptoms of appendicitis partly accompanied by diarrhoea were recorded in 20 patients. Gastric and abdominal disturbances without diarrhoea were present in 26 patients. Only one case of *Y. enterocolitica* positive erythema nodosum was associated with gastroenteritis. Rheumatic symptoms were recorded in 5 patients. Carditis or suspected carditis and arthralgia in patients positive for *Y. enterocolitica* were often accompanied by pyrexia, upper respiratory catarrh, and enteritis. Cases with miscellaneous diagnosis were included in Table V as "other extraintestinal

Table III

Serogroup distribution of Y. enterocolitica in humans in Hungary, 1969-1974

Public health laboratories	Serogroups							Total
	O1, 2a, 3	O3	O5	O6	O9	O10	O15	
Baranya		1						1
Békés		45		1				46
Borsod-Abaúj-Zemplén		45						45
Csongrád		6	1				1	8
Fejér		29						29
Győr-Sopron		5						5
Hajdu-Bihar	2	5						7
Heves		40						40
Komárom		4						4
Nógrád		8						8
N.I.P.H.*		197						197
Somogy		14						14
Szabolcs-Szatmár		1						1
Szolnok		4						4
Vas		19				1		20
Veszprém		428			2			430
Budapest		442			4			446
László Hospital**		36						36
Szeged		14						14
Total	2	1343	1	1	6	1	1	1355

* National Institute of Public Health, Budapest

** Budapest

symptoms". Among these a case of chronic prostatitis caused by *Y. enterocolitica* O9 deserves mentioning. *Y. enterocolitica* positive patients in whom the clinical symptoms (urticaria bullosa, cholelithiasis, rectal adenocarcinoma, etc.) were obviously not associated with the agent, were included in this group.

Table V demonstrates that yersiniosis, especially its enteric form, affects mainly 1 to 9-year-old children. Symptomless excretors (23.1% of all positive persons) were detected in the course of screening food handlers, nursery personnel, children to be admitted to nurseries and persons in the environment of patients with yersiniosis. Symptomless excretors, in contrast to patients with enteric complaints, were evenly distributed in all age groups. Table VI shows that yersiniosis occurred somewhat more frequently in males than in females (56.3 and 43.3%, respectively).

Table IV

Antibody titres in patient's sera against Y. enterocolitica O3 and O9 antigens

Years	Antigen O3 (strain Wbdx)												Total
	< 10*	10	20	40	80	160	320	640	1280	2560	5120	10240	
1969-1971	465	17	13	11	3	1	8	9	7	3	—	—	537
		495		11					31				537
		92.2%		2.0%					5.8%				100.0%
1972	225	14	18	15	26	27	22	12	8	6	12	3	388
		257		15					116				388
		66.2%		3.9%					29.9%				100.0%
1973	378	11	27	42	62	51	45	29	25	27	20	—	717
		416		42					259				717
		58.0%		5.9%					36.1%				100.0%
1974	323	5	13	28	15	33	22	25	28	28	30	—	550
		341		28					181				550
		62.0%		5.1%					32.9%				100.0%
Total	1391	47	71	96	106	112	97	75	68	64	62	3	2192
		1509		96					587				2192
		68.8%		4.4%					26.8%				100.0%

* Reciprocal of serum dilutions

Table VII shows the distribution of serogroups according to clinical symptoms. It is evident that members of group O3 were responsible for every clinical form of yersiniosis.

Antibiotic sensitivity. Susceptibility testing *in vitro* showed that 584 human strains were uniformly resistant to penicillin, oxacillin, ampicillin, novobiocin, spiramycin, oleandomycin, vancomycin and lincomycin. All strains were sensitive to chloramphenicol, neomycin, polymyxin B and streptomycin as well as to gentamicin, colistin and kanamycin. In 1973, all strains were sensitive to tetracycline; in 1974, 0.2% showed resistance to this antibiotic. Some authors have reported the occurrence of strains moderately sensitive to certain broad spectrum antibiotics; apart from these, our findings agree well with the literary data. Nalidixic acid and especially trimethoprim-sulphamethoxazole and nitrofurantoin are said to be highly effective [38]. Recently we have examined 20 *Y. enterocolitica* strains on a modified Mueller-Hinton medium [39] and found that all of them were sensitive to sulphonamides and trimethoprim-sulphamethoxazole.

(1969-1974)

Antigen O9 (strain 5385)												Total
<10	10	20	40	80	160	320	640	1280	2560	5120	10240	
449	10	24	18	13	9	8	5	1	—	—	—	537
	483		18					36				537
	89.9%		3.4%					6.7%				100.0%
375	3	6	1	—	—	1	1	—	—	1	—	388
	384		1					3				388
	98.9%		0.3%					0.8%				100.0%
694	4	4	—	3	6	3	2	1	—	—	—	717
	702		—					15				717
	97.9%							2.1%				100.0%
520	2	5	6	7	5	4	—	—	—	1	—	550
	527		6					17				550
	95.8%		1.1%					3.1%				100.0%
2038	19	39	25	23	20	16	8	2	—	2	—	2192
	2096		25					71				2192
	95.7%		1.1%					3.2%				100.0%

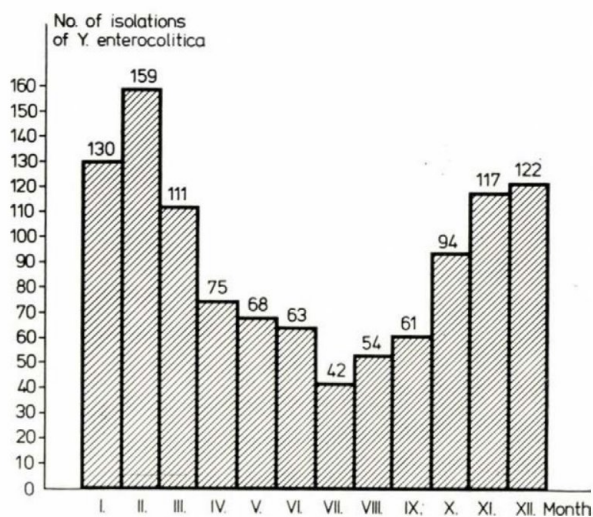


Fig. 2. Monthly distribution of *Y. enterocolitica* isolates identified in the National Institute of Public Health, Budapest, 1969-1974

Table V

Distribution of patients and excretors according to main clinical symptoms and age groups (1969-1974)

Age groups	Intestinal symptoms			Extraintestinal symptoms			Symptomless excreters	Unknown	Total	
	Enteritis	Appendicular	Other gastro-intestinal complaints	Erythema nodosum	Rheumatic complaints	Other			No.	Per cent
0	21					2	4	3	30	2,7
1-2	112				1	7	32	16	168	15,3
3-5	147	5	6			8	51	26	243	22,2
6-9	81	3	1		1	1	11	14	112	10,2
10-14	47	3	9		1	3	12	11	86	7,9
15-19	39	2	1			2	21	7	72	6,6
20-29	62	1	5		2	1	34	10	115	10,5
30-39	38	2	3				29	16	88	8,0
40-49	29		1	1		2	20	11	64	5,8
50-59	6						11	5	22	2,0
60-	9						15	3	27	2,5
Unknown	28	4				3	13	21	69	6,3
Total number	619	20	26	1	5	29	253	143	1096	
Per cent	56.5	1.8	2.4	0.1	0.5	2.6	23.1	13.0		100.0

Seasonal incidence. As seen in Fig. 2, yersiniosis shows an autumn-winter peak. In decreasing at the end of spring and exhibiting a minimum incidence in summer, the disease differs from common enteric infections.

Animal reservoirs. Strains isolated from animals are presented in Table VIII. The majority of isolates originated from pigs. The fact that serogroup O3 predominated not only in humans but also in animals, allows some conclusions to the source of human infections.

Table VI

Sex distribution of Y. enterocolitica positive persons (1969-1974)

Sex	Persons	
	Number	Per cent
Male	617	56.3
Female	475	43.3
Unknown	4	0.4
Total	1096	100.0

Table VII

Serogroups of Y. enterocolitica in relation to clinical symptoms (1969-1974)

Clinical symptoms	Serogroups							Total
	O1, 2a, 3	O3	O5	O6	O9	O10	O15	
Enteritis		616			3		1	620
Appendicitis		19			1			20
Other gastrointestinal complaints		26						26
Erythema nodosum		1						1
Rheumatic complaints		5						5
Other symptoms		28			1			29
Symptomless excretors	2	248	1		1	1		253
Unknown		141		1				142
Total	2	1084	1	1	6	1	1	1096

Table VIII

*Incidence of Y. enterocolitica in animals (1969-1974)**

Animal species	Serogroups			Total
	O3	O4	O5	
Pig	39			39
Cat	6			6
Dog	7			7
Hen	3		1	4
Duck		1	1	2
Hare	1			1
Total	56	1	2	59

* Strains identified in the National Institute of Public Health, Budapest

Discussion

The effectiveness of selective media for the cultivation of *Y. enterocolitica* was compared by NILÉHN [40]. In this laboratory we examined home-made bismuth sulphite, desoxycholate citrate, eosin methylene blue, brilliant green and blood agar by inoculating strains O3 and O9 in pure cultures and in faecal suspensions. Blood agar gave the best results both at room temperature and at 37°C. Although eosin methylene blue allowed the recovery of somewhat more bacteria than did DC agar, in routine work the latter was more advantageous, partly because yersiniae produced characteristic colonies on it, partly because of its general use as a highly selective faecal culture medium. On DC agar the minimum inoculum for the growth of one colony was 10 yersinia cells

if seeded in pure culture and 10^2 cells if the organism had been mixed to a faecal suspension.

The antigenic structure of our strains was determined by slide agglutination on the basis of WINBLAD's scheme [41, 42], supplemented by WAUTERS *et al.* [43-45] to contain 34 O and 19 H antigens. (Advocating the earlier classification system of KNAPP and THAL [46], in 1973 KNAPP [47] recommended a scheme based on six O antigenic groups.)

One hundred and fifty isolates, as representatives of our strains isolated in 1973, were sent to Professor H. H. MOLLARET. He confirmed our results of serogrouping and classified our O3 strains into NILÉHN's [10] biotype 4 and into NICOLLE's [48, 49] phage types 9/a (12 strains), 11 (1 strain) and 8 (92 strains).

Antibody production is fairly frequent in human yersiniosis. The sera of our patients with suspected or verified yersiniosis showed positive titre in 26.8% against serogroup O3. Antigen O9 may react with *Brucella* antibodies, especially in countries where brucellosis still occurs. KNAPP [47] and LYSY and KNAPP [50] observed that certain *Y. enterocolitica* OH antigens agglutinate in sera containing antibodies to *Morganella*. The relationship of *Y. enterocolitica* O9 and O17 to *Morganella* O43 and O44, respectively, was confirmed by VELIN and RAUSS [51]. Accordingly, sera reacting with *Y. enterocolitica* antigen O9 should be checked with *Brucella* and *Morganella* O43 antigens.

Studies on the antibiotic sensitivity of *Y. enterocolitica* [22, 38, 52] have recently been supplemented by HAUSNEROVÁ *et al.* [53], who showed that penicillin and its semisynthetic broad-spectrum derivatives were ineffective against *Yersinia*; they isolated two tetracycline resistant strains and found that colistin, penicillin and its broad-spectrum derivatives offered a reliable aid for differentiating *Y. pseudotuberculosis* and *Y. enterocolitica*. The therapeutic effectiveness of trimethoprim-sulphamethoxazole was described by GUTMAN *et al.* [54] and TOMA and LAFLEUR [30].

It is of interest to compare our observations on the clinical forms of yersiniosis described in detail earlier [55] with data reported from other countries. In our material the predominance of enteritis (56%) may be explained by the fact that, in order to diagnose the causative agent of enteric diseases faecal samples are usually sent for laboratory examination. In contrast, in the case of surgical patients, bacteriological cultivation is less frequently asked for. In Czechoslovakia [26] 80.9% of *Y. enterocolitica* isolations were made from patients with enteritis; other Czechoslovak data also show the predominance of enteric forms [56]. Diarrhoeal cases are the most frequently encountered in yersiniosis both in the Democratic [57] and in the Federal [58] Republic of Germany. In Belgium, VANDEPITTE *et al.* [59] showed 523 (81.8%) gastroenteric, 97 (15.2%) appendicular and 19 other forms of yersiniosis in 639 patients, whereas $\frac{2}{3}$ of VAN OYE's [60] cases were characterized by enteric

symptoms. In the Netherlands, the isolates of ESSEVELD and GOUDZWAARD [61] were mostly cultured from patients with enteritis. MOLLARET [62] in France reported an overwhelming majority of enteric cases in all age groups. Between 1965 and 1971 in Sweden WINBLAD [63] examined 718 patients with positive serological and cultural findings for *Y. enterocolitica* and found the following distribution: 184 surgical cases (acute terminal ileitis, mesenterial lymphadenitis, pseudoappendicitis), 175 erythema nodosum, 162 enteritis, 78 arthritis and 55 pyrexia; all in all 62.3% of the culturally confirmed yersinioses exhibited enteric symptoms. In Finland, 95 out of 304 patients had abdominal complaints or diarrhoea, and 80 appendicitis; in 97 patients with erythema nodosum and in 101 with arthritis the disease was usually preceded by gastrointestinal disorders [29]. In Canada, TOMA and LAFLEUR [30] reported 244 gastroenteritis in 256 patients. Between 1968 and 1972, bacteriologists at the U.S. Center for Disease Control identified 29 strains, including 6 isolates from blood; only 4 of these cultures originated from faeces [17]. Half of their strains corresponded to "Bacterium enterocoliticum" (*Y. enterocolitica* O8); they failed to isolate the serogroup O3 and O9 prevalent in Europe. In South Africa [18], 15 out of 34 yersiniosis patients complained of diarrhoea and vomiting.

Our data showing a relatively high incidence of symptomless excretors are based on examination of food handlers, children to be admitted to nurseries and of healthy individuals in the environment of patients. The incidence of symptomless excretors may, in reality, be lower, since a history of enteric disease could not be excluded in all persons. In turn, the clinical symptoms might have been due to other causes in some patients with positive *Y. enterocolitica* finding.

In agreement with other authors [59, 62], yersinia enteritis affected frequently children between 1 and 9 years of age (47%). Our data for sex distribution closely coincided with those of VANDEPITTE *et al.* [59].

The role of *Y. enterocolitica* in rheumatic arthritis is well known [64-66]. Animal sources of human yersinia infections have extensively been investigated. In Hungary, animal strains were cultured mainly from the environment of patients [67, 68]. KUBINYI [69] isolated *Y. enterocolitica* from the faeces of 22 pigs not connected with human infections. Observations indicate that the main reservoir of *Y. enterocolitica*, as that of *Salmonella*, is the pig [18, 30, 59, 61, 70-72]. The portal of entry of the agent is mostly the mouth; a possible introduction via the eye or the respiratory tract has also been assumed. The role of water in the spreading of yersinia has been suggested in Norway [73]. Food may also convey the infection: in one of our *Y. enterocolitica* outbreaks the epidemiological investigation suggested food-borne infection (the incriminated food was not available).

The autumn-winter peak of the disease in Hungary is in good agreement

with reports from other countries: October–January in Belgium, November in Sweden, September–November in Finland, December–March in Czechoslovakia and November–March in Romania.

In Hungary, sporadic cases and family outbreaks are the most frequent [74]. Epidemic incidence, including a sudden outbreak in a nursery, was also observed [75–77]. In Japan, ASAKAWA *et al.* [19] reported on epidemics involving great numbers of children and adults in two schools and in a nursery. TOIVANEN *et al.* [78] described iatrogenic yersinia infections in patients and nurses in two wards of a hospital. From observations in epidemics it would appear that the incubation period of enteric yersiniosis is about 10 days and the contagiousity of the infection is low.

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BUOYANT DENSITY OF GOOSE PARVOVIRUS STRAIN "B"

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Summary. For the buoyant density of goose parvovirus strain "B", a value of 1.380 g/ml was obtained by isopycnic CsCl gradient centrifugation.

Parvoviruses are small, icosahedral non-enveloped DNA-containing viruses. All of them band in isopycnic CsCl gradients in the range of 1.38 to 1.46 g/ml. They can be divided into two subgroups: those replicating only in the presence of helper viruses (adeno-associated-viruses), and others, being capable of multiplying without helper viruses. The replication processes of some of the latter are facilitated in rapidly growing cell cultures.

The "B" virus strain has been isolated in Hungary from young gosling carcasses [1] and identified as a possible member of the parvovirus group [2]. Its replication has been shown to depend on the growing cycle of cell cultures used for cultivation [3]. Several authors have isolated from young goslings virus strains of similar properties [4, 5]. Cross-neutralization tests revealed a close antigenic relationship between the "B" virus strain and those isolated in France, the Netherlands and the USSR [6, 7].

This study had been prompted by the lack of data on buoyant density values of these virus strains; as their representative, the "B" virus strain was chosen.

Materials and methods

The "B" virus strain was propagated in goose embryo fibroblast cell culture as described previously [2]. One thousand and two hundred ml of tissue culture harvests of "B" virus strain of 37th tissue culture passage were concentrated by ultracentrifugation (Janetzki VAC 60 ultracentrifuge, 8 × 30 ml rotor, at 5°C, 36 000 rpm for 3 hr). The virus pellets were resuspended in 6 ml of double distilled water. Solid CsCl was added to the virus suspension to obtain an average density of 1.42 g/ml, then it was placed into two tubes of an SW 41 Ti rotor and filled up with mineral oil. Centrifugation was performed in a Beckman-Spinco L2-65B ultracentrifuge at 25°C, 35 000 rpm for 24 hr. The gradients were fractionated by puncturing a hole in the bottom of the tubes and collecting 0.10 ml fractions. The specific gravity of each fraction was calculated from the refractive index measured by means of an ABBE refractometer at 25°C. Fractions of 1.35–1.44 g/ml density (peak of infectivity at 1.36–1.41 g/ml) were pooled and adjusted by adding solid CsCl to an average density of 1.40 g/ml. This partially purified virus suspension was ultracentrifuged in an SW 41 Ti rotor at 35 000 rpm at 25°C for 48 hr. Fractions of 0.15 ml were collected as above. After determining the specific gravity of

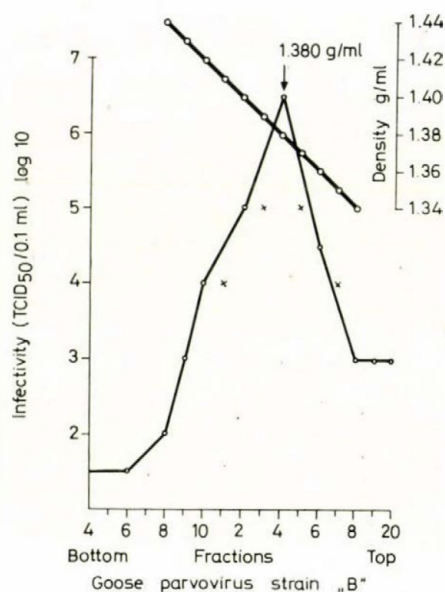


Fig. 1. Isopycnic gradient centrifugation of goose parvovirus strain "B" in CsCl for 48 hr

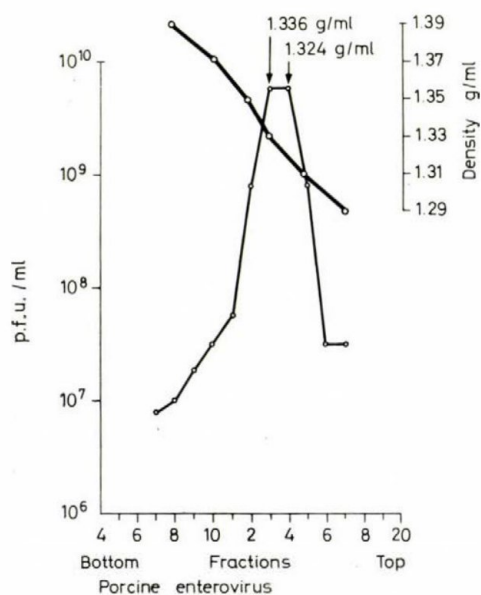


Fig. 2. Isopycnic centrifugation of porcine enterovirus strain 5-DVIII in CsCl for 48 hr

each fraction, Hanks' solution was added to make a 1 : 10 dilution. These samples were stored at -20°C until assayed for infectivity.

As reference strain, the porcine enterovirus strain 5-DVIII [8] propagated in PK-15 cell line was used. It underwent a similar procedure as the goose parvovirus strain "B", with suitable modifications as to the initial CsCl density.

Results and discussion

As it is shown in Figs 1 and 2, the majority of the infective particles of goose parvovirus strain "B" was bound in the CsCl gradient at a density of 1.380 g/ml. This value confirmed the earlier suggestion [2] that the goose virus isolate designated as strain "B" should be regarded as a member of the parvovirus group.

The value of buoyant density obtained in this study for porcine enterovirus roughly corresponds to that described earlier [9].

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ZUR SEROLOGIE DER STAPHYLOKOKKEN VON PATIENTEN VERSCHIEDENER KLINIKEN

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(Eingegangen am 30. Juli 1975)

H. OEHRING and R. OEHRING (*Institute of Medical Microbiology, Friedrich Schiller University, Jena, G.D.R.*): **Incidence of Staphylococcal Serotypes in Different Hospitals.** A total of 409 *Staphylococcus aureus* strains were classified with 17 typing sera into 16 groups and 14 types. Strains with the formula $a b^+ c e$ and $h^+ i k l$ were most frequently met with. The majority of strains isolated from typical staphylococcal diseases contained antigens e^+ , h^+ and/or h^{++} whereas those associated with atypical infections showed antigens b^{++} and o most frequently. Classification by Oeding and Williams' scheme yielded a less wide variety of serological units: most strains fell into group 4; spontaneously agglutinating and group 1, 2 and 3 strains were next in order.

Zusammenfassung. Es wird über die Ergebnisse serologischer Studien an 409 *Staphylococcus aureus* Stämmen berichtet. Sie wurden mit dem Ziel einer Differenzierung unter dem Aspekt ihrer klinischen Herkunft durchgeführt. Untersuchungen mit 17 Faktorensereen ergaben 16 Gruppen und 14 Typen, deren häufigste in allen Kliniken zu finden waren. An erster Stelle lagen Stämme mit den Formeln $a b^+ c e$ und $h^+ i k l$. Durch statistische Berechnungen konnten signifikante Häufigkeitsdifferenzen in der Verteilung der Gruppen und Typen festgestellt werden. Die Einteilung nach OEDING und WILLIAMS ergab nicht eine solche Vielfalt der serologischen Ergebnisse. Die meisten Stämme gehörten der Gruppe 4 an, welche von den spontan-agglutinierenden Stämmen sowie den Gruppen 1, 2 und 3 gefolgt waren. Am deutlichsten traten die Unterschiede zutage, wenn das isolierte Vorkommen der Antigene analysiert wurde. Dabei stellten sich mehrere abweichende serologische Muster dar. Diese waren durch bestimmte Leitantigene charakterisiert. So besaßen Stämme der Einrichtungen mit typischen Staphylokokkenerkrankungen die Antigene e^+ , h^+ und/bzw. oder h^{++} . In anderen Kliniken, deren Patienten an Infektionen litten, welche nicht zu den typischen gerechnet werden, waren die Antigene b^{++} und o oft vorhanden. Ihre Anwesenheit war durch das serologische Bild der Erreger aus typischen Infektionen bedingt.

Über das epidemische Auftreten von Staphylokokkenerkrankungen in Kliniken und Krankenhäusern liegen unzählige Berichte vor. In ihnen wird über die Brauchbarkeit mikrobiologischer Typisierungsverfahren bei der Aufdeckung von Infektionsquellen und -wegen berichtet. Eine der Methoden, die weitgehende Differenzierungsmöglichkeiten bietet, ist die Serologie.

Untersuchungen an Stämmen von Patienten, welche die Infektionen außerhalb von Kliniken und Krankenhäusern erwarben, wurden seltener durchgeführt. Es war jedoch von Interesse zu wissen, ob sich bei denjenigen Stämmen serologische Unterschiede nachweisen ließen, die aus pathologischen Prozessen stammten und nicht im Zusammenhang mit einem epidemischen Geschehen standen. Durch die Typisierungen sollte versucht werden, solche Merkmale aufzufinden, mit deren Hilfe die Stämme von Patienten der verschiedensten Kliniken abgegrenzt werden könnten.

Material und Methoden

Stämme. Zur Untersuchung gelangten 409 *Staphylococcus aureus* Stämme, deren Herkunft aus Tabelle I ersichtlich ist.

Tabelle I

Herkunft der untersuchten S. aureus-Stämme

Klinik	Anzahl der Stämme
Chirurgische Klinik	65
Chirurgische Poliklinik	79
Kinderklinik (Eiterstämme)	63
Kinderklinik (fäkale Stämme)	76
HNO-Klinik	54
Medizinische Klinik	24
Frauenklinik	18
Sonstige	30
Insgesamt	409

Tabelle II

Absorptionsschema

Faktoren-serum	Immun-serum	Absorption mit Stamm
a	3647	F 21
b*	2095	2253
b**	2095	2253 nativ und 2095 autoklaviert
BH	2095	2253 und 3189
c	3647	1503 und 3189
e	1503	3647
e'	1503	3647 und Cowan I
f	3189	2095
h*	17 A	1503
h**	17 A	1503 nativ und 17 A autoklaviert
i	F 21	1503 und 3647
k	S 356	Cowan I und F 21
l	A 4	D 124
m	F 21	Wood 46 und 3647
n	Cowan III	2095 und F 21
o	D 124	D 86
p	D 142	D 86 und D 124

* nach OEDING [3]

** nach GRÜN [9]

Die Titer der Faktorensereen betragen nach der Absorption 1 : 2-1 : 16

Die Stämme wurden von Patienten isoliert, die an nachstehenden Infektionen litten: 1. Mastitis (M) 16; 2. Infektionen des lymphatischen Systems (L) 6; 3. Infektionen des Bewegungsapparates (B) 9; 4. Konjunktivitis (C) 10; 5. Otitis (O) 36; 6. Infektionen des Urogenitalsystems (U) 19; 7. Infektionen des Darmtraktes (D) 18; 8. Infektionen des Respirationstraktes (R) 40; 9. Wundinfektionen (W) 19; 10. Panaritium (Pa) 24; 11. Furunkel (Fu) 44; 12. Phlegmonen, Pyodermien, Abszesse (PhA) 77; 13. Herkunft unbekannt (X) 15; 14. Säuglingsdyspepsie (F) 76; Insgesamt 409.

Die Stämme mit bekannter Herkunft von Nr. 1–12 faßten wir schließlich nach anderen Gesichtspunkten zu folgenden 3 Gruppen zusammen. 1. Infektionen der Haut oder von dort ausgehende Prozesse (H) 180; 2. Infektionen der Schleimhäute oder von dort ausgehende Prozesse (S) 104; 3. Infektionen der inneren Organe (P) 34; Insgesamt 318.

Seren. Die serologische Differenzierung nahmen wir mit der zuerst von OEDING [1–5] angegebenen und von GRÜN [6–9] und PULVERER [10] modifizierten Technik vor. Sie erfolgte mit Hilfe von 17 selbst hergestellten absorbierten Faktorenseren.

Zur Herstellung wurden formalinbehandelte Staphylokokken verwendet. Die Erreger waren in 50 ml 1%iger Dextrose- oder Fleischbouillon angezüchtet worden. Das Formalin wurde durch mehrmaliges Auswaschen entfernt. Der Keimgehalt der Vaccine lag bei $3-4 \times 10^9$ Keimen/ml. Die Immunisierung erfolgte in drei Serien 3–4 Wochen lang. Nach Bestimmung des Agglutinititers nahmen wir die Herstellung der Faktorensereen entsprechend dem folgenden Schema vor. Dafür waren nachstehende Immunsereen und Staphylokokkenstämme erforderlich (Tabelle II).

Agglutination. Bei der Testung gingen wir so vor, daß 5 hr alte, bei 40°C gewachsene Kulturen des zu testenden Stammes in je einem Tropfen jedes der o.g. Seren auf Tüpfelplatten verrieben wurden. Nach 2 hr Aufenthalt bei 37°C in einer feuchten Kammer erfolgte die Ableseung mit einer Lupe von 6facher Vergrößerung. Die Einteilung in Gruppen und Typen richtete sich nach den Angaben von GRÜN [8], OEDING [3] und OEDING und WILLIAMS [11].

Statistische Methode. Aufgrund der Typisierung mit mehreren Faktorenseren, der differentiellen Herkunft der Stämme und der Auswertung unter verschiedenen Gesichtspunkten war mit einer großen Zahl von Einzelergebnissen zu rechnen, die nur durch statistische Berechnungen zu sichern waren. Sie erfolgten mit Hilfe der Formel zur Prüfung einer Hypothese über die Differenz zweier Häufigkeitsziffern [12]:

$$t = \frac{D}{s_D} = \frac{f_1 - f_2}{\sqrt{pq \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

Ergebnisse

Die Resultate der serologischen Untersuchungen sind in Abb. 1–4 dargestellt. Wenn sich dort auch des öfteren auffällige Häufungen einer Formel bzw. eines Merkmals feststellen lassen, so haben diese nicht in jedem Falle eine signifikante Bedeutung. Lediglich die Resultate der statistischen Berechnungen konnten hier gesicherte Unterschiede aufdecken. Um zu einer übersichtlichen Charakterisierung zu kommen, wurden für jede Klinik nur diejenigen Formelbilder besonders markiert, welche signifikant öfter bzw. seltener bei einer oder mehreren anderen Einrichtungen herausragten.

Die Entscheidung, ob das Resultat der statistischen Untersuchung, d. h. der erhaltene t-Wert in den Signifikanzbereich fällt, ist neben der Differenz (D) der relativen Häufigkeiten ($f_1 - f_2$) — letztere graphisch dargestellt — insbesondere von den Umfängen der beiden verglichenen Versuchsreihen ($n_1; n_2$) abhängig. Wenn diese groß sind, kann eine relativ kleine Differenz der beiden Häufigkeiten statistisch signifikant sein. Demgegenüber kann eine größere

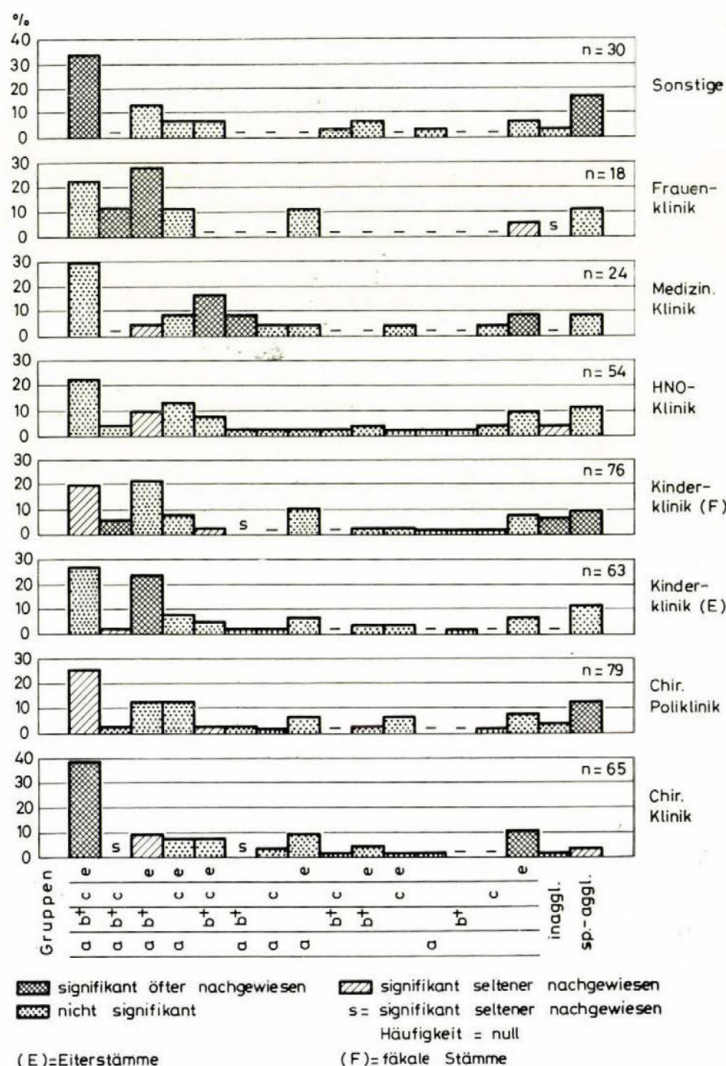


Abb. 1. Das Vorkommen serologischer Gruppen der *S. aureus*-Stämme isoliert aus pathologischen Prozessen von Patienten verschiedener Kliniken

Differenz bei geringen Umfängen der beiden Serien noch im Bereich des Zufälligen liegen.

Abb. 1 zeigt die Verteilung der Gruppen nach den Angaben von GRÜN [8]. Wir konnten die 407 Eiter- und Entzündungsstämmen mit Hilfe der Antigene a, b⁺, c und e in 16 Gruppen, ohne die spontanagglutinablen Stämme, unterteilen, von denen mit 26,9% die Formel a b⁺ c e am häufigsten war.

Weiter war es möglich, zwischen den Patientenstaphylokokken der verschiedensten Einrichtungen Differenzen im Vorkommen bestimmter serologi-

scher Gruppen zu erkennen, die häufigsten Formelbilder waren jedoch in allen Kliniken anzutreffen. Es bestand kein Hinweis dafür, daß die Staphylokokken von Patienten einer bestimmten Einrichtung durch die exzessive Häufung bzw. das Fehlen einer bestimmten serologischen Gruppe definiert waren. Vielmehr traten die wichtigsten Formeln, deren Häufigkeit im gesamten Stamm-Material 8,1% und mehr betrug, unter den Patientenstaphylokokken aller Einrichtungen auf.

Ein Vergleich der Versuchsreihen untereinander in bezug auf die signifikanten Serogruppen zeigte, daß sich keine mit einer anderen vollständig deckte. Dabei verhielten sich die Stämme der Chirurgischen Klinik gegenüber den Staphylokokken von poliklinischen Patienten der gleichen Einrichtung, die fäkalen Stämme der Kinder- sowie der Frauenklinik nahezu konträr. Zu beachten ist ferner, daß Stämme der zuletzt genannten Einrichtung gegenüber Staphylokokken von Patienten mit internen Erkrankungen (Med. Klinik) einige Diskrepanzen zeigten.

Durch die Antigene h^+ , i , k und l gelang es, 14 Typen ohne die spontan-agglutinierenden Stämme abzugrenzen, wie aus Abb. 2 hervorgeht. Hier dominierten Stämme der Formel $h^+ i k l$, deren Vorkommen sich auf 38,1% belief. Es fiel auf, daß die Staphylokokken von Patienten der aufgeführten Kliniken bezüglich der serologischen Typen Unterschiede in der Häufigkeit ihres Auftretens besaßen. Die wichtigsten Formeln, deren Vorkommen insgesamt im Stamm-Material 8,8% und mehr ausmachten, fanden sich jedoch in allen Beobachtungsreihen vor. Berücksichtigte man lediglich Typen, für die signifikante Abweichungen nachgewiesen worden waren, so ergab sich keine Identität zwischen zwei verglichenen Beobachtungsreihen. Sehr deutlich wichen die Staphylokokken von Patienten der Chirurgischen Klinik und jenen aus Stuhlproben der Kinderklinik im Hinblick auf drei Typen ab. Nach Gegenüberstellung anderer Serien fanden sich größere Häufigkeitsdifferenzen jeweils nur bei einem Typ. Einzelheiten sind der Abb. 2 zu entnehmen.

Am auffälligsten waren drei signifikante Häufungen. Sowohl in der betreffenden Beobachtungsreihe als auch im gesamten Untersuchungsmaterial entfielen auf sie relativ die meisten Stämme. Bei den nachstehend genannten Kliniken handelte es sich um folgende Typen: Chirurgische und Kinderklinik (E): $h^+ i k l$; Frauenklinik: $h^+ k l$.

In diesem Zusammenhang war es von Interesse, wie hoch sich der Anteil solcher Typen in jeder Beobachtungsreihe belief, bei denen signifikante Häufigkeitsdifferenzen vorhanden waren. Wenn man diejenigen berücksichtigte, die öfter nachgewiesen wurden und die Typen, die seltener auftraten, dazu rechnete, machten sie 8,3–66,2% im Material der einzelnen Einrichtungen mit Ausnahme der HNO-Klinik aus.

Wesentlich andere Resultate, welche aus Abb. 3 zu ersehen sind, erhielt man, wenn der Differenzierung die Gruppeneinteilung nach OEDING und

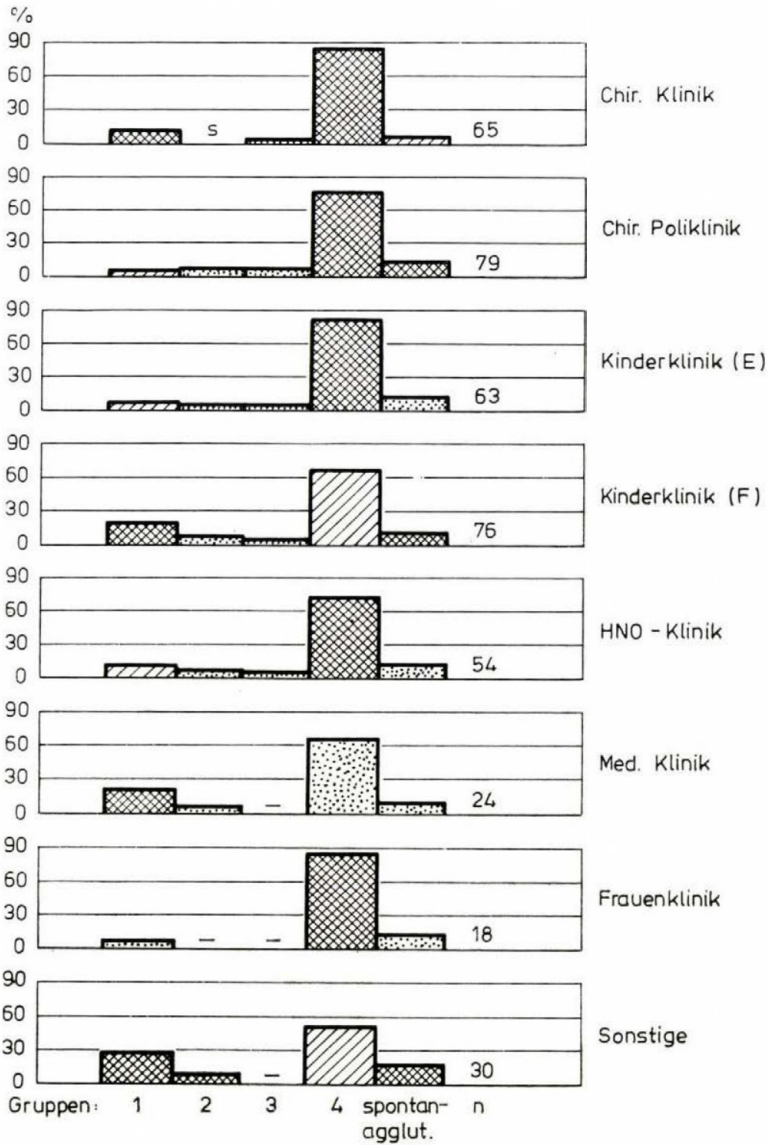


Abb. 3. Das Vorkommen serologischer Gruppen nach OEDING und WILLIAMS der *S. aureus*-Stämme aus pathologischen Prozessen von Patienten verschiedener Kliniken

Bei allen anderen Gruppen stellten wir keine eindeutigen Korrelationen fest.

Im Gegensatz zu den Gruppen war die Anzahl der charakteristischen Typen wesentlich geringer. Es handelte sich bei den meisten Krankheitsbildern, mit Ausnahme der Infektionen des Urogenitaltraktes nur um eine Formel, was auch auf die Einteilung unter Berücksichtigung der Kliniken zutraf. Der Versuch einer Zuordnung der signifikant öfter angetroffenen Typen zu Diagnosen und Kliniken deckte nachstehende Beziehungen auf:

Typ	Klinik			Diagnose/Fundort
h ⁺ i k l	Chirurgische Klinik			Bewegungsapparat (B)
	Kinderklinik (E)			Furunkel (Fu)
h ⁺ i l	Medizinische Klinik			Urogenitaltrakt (U)
h ⁺ k l	Frauenklinik			Mastitis (M)
h ⁺ l	Sonstige			Konjunktivitis (C)

Bei der Gruppenbildung nach OEDING und WILLIAMS [11] fanden wir im wesentlichen die gleichen Korrelationen zwischen Krankheitsbildern und Klinik. Sie sind aus der folgenden Darstellung ersichtlich.

Gruppe	Klinik	Diagnose/Herkunft
1	Kinderklinik (F)	Säuglingsdyspepsie (F)
		Schleimhautinfektion (S)
	Medizinische Klinik	Urogenitaltrakt (U)
		Darmtrakt (D)
4	Chirurgische Klinik	Schleimhautinfektion (S)
		Panaritium (Pa)
		Phlegmonen, Pyodermien
		Abszesse (PhA)
	Chirurgische Poliklinik	
	Kinderklinik (E)	

Aus dem Gesagten geht hervor, daß die Häufung bestimmter Gruppen und Typen der *S. aureus*-Stämme im Untersuchungsmaterial einiger Kliniken durch das Vorherrschen derselben bei typischen Infektionen zu erklären ist. Nachdem es sich erwiesen hatte, daß eine Differenzierung von Staphylokokkenstämmen unterschiedlicher Kliniken gelang, wenn man die verschiedensten Antigenkombinationen berücksichtigte, sollte im folgenden das isolierte Vorkommen der einzelnen Antigene analysiert werden.

Die Resultate dieser Untersuchungen sind in Abb. 4 graphisch dargestellt. Auf eine Erörterung der relativen Häufigkeiten haben wir verzichtet und vielmehr die signifikanten Abweichungen der Darstellung zugrunde gelegt. Da es nicht nur von Interesse war, ob ein Antigen in einer Untersuchungsreihe öfter als woanders vorkam, sondern auch diejenigen eine Bedeutung besaßen, die seltener nachgewiesen wurden bzw. fehlten, haben wir Alternativen durch unterschiedliche Symbole gekennzeichnet.

Bei der Betrachtung der Abb. 4 ist zu erkennen, daß die Staphylokokken keiner Klinik (Versuchsreihe) mit einer anderen identisch waren. Zwar gab es Faktoren, bei denen gesicherte Abweichungen nicht vorhanden waren, jedoch existierte eine wechselnde Anzahl solcher Antigene, für die sich echte Diffe-

renzen nachweisen ließen. Es fiel auf, daß die Stämme der HNO, Medizinischen und Chirurgischen Klinik durch relativ viele Antigene mit signifikanten Unterschieden charakterisiert waren, wogegen diese Anzahl bei anderen, z. B. sonstige Einsender, Chirurgische Poliklinik, Frauen- und Kinderklinik (Eiterstämme) darunter lag. Weiter bemerkten wir, daß es Stämme gab, die durch das Dominieren solcher Antigene imponierten, die öfter vorkamen (Frauen-, Chirurgische und Medizinische Klinik), während sich andere dadurch

	Serologische Antigene																
	a	b ⁺	b ⁺⁺	BH	c	e	e'	f	h ⁺	h ⁺⁺	i	k	l	m	n	o	p
Chir. Klinik			■	■	■	■				■	■	■		■			■
Chir. Polikl.			■			■					■	■		■	■		■
Kinderkl. (E)			■				■		■	■		■			■	■	■
Kinderkl. (F)			•	•	■	■	•	•		•	■	■			•	•	•
HNO Klinik	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Med. Klinik			■	■	■	■	■	■			■					■	■
Frauenkl.	■					■	■		■			■		■	■		
Sonstige			■				■			■	■	■					■

■ signifikant öfter nachgewiesen

▨ signifikant seltener nachgewiesen (bzw. nicht vorhanden)

• nicht geprüft + nach OEDING ++ nach GRÜN

(E)=Eiterstämme (F)=fakale Stämme

Abb. 4. Antigenmuster der *S. aureus*-Stämme isoliert aus pathologischen Prozessen von Patienten verschiedener Kliniken

auszeichneten, daß eine größere Anzahl von Antigenen seltener vorhanden war oder fehlte [Kinderklinik (F), HNO und Chirurgische Poliklinik, sonstige Einsender]. Bereits oben hatten wir festgestellt, daß sich keine der verglichenen Beobachtungsreihen miteinander deckte, da eine mehr oder weniger große Anzahl von Differenzen hinsichtlich der Häufigkeit bestimmter Antigene existierte. Es können hier jedoch nicht alle Einzelheiten erwähnt werden. Sie sind der Abb. 4 zu entnehmen. Lediglich die stärksten Abweichungen wollen wir besprechen. Diese bestanden zwischen der 1. Kinderklinik (Eiterstämme) und der Medizinischen Klinik sowie sonstigen Einrichtungen; 2. HNO-Klinik und der Chirurgischen sowie Frauenklinik; 3. Medizinischen Klinik und der Kinderklinik (Eiterstämme).

Eindrucksvoll war auch das Bild, wenn man lediglich diejenigen Antigene mit signifikant öfter nachgewiesenen Vorkommen schriftlich fixierte. Man erhält dabei folgende Darstellung (Tabelle III).

Tabelle III

Zusammensetzung der *S. aureus*-Stämme aus serologischen Antigenen,
die besonders häufig nachgewiesen wurden

Klinik	Antigene																
	a	b ⁺	b ⁺⁺	BH	c	e	e ⁺	f	h ⁺	h ⁺⁺	i	k	l	m	n	o	p
Chirurgische Klinik					c	e	e ⁺			h ⁺⁺	i	k		m			p
Chirurgische Poliklinik							e ⁺					k			n		p
Kinderklinik (E)									h ⁺	h ⁺⁺		k					
Kinderklinik (F)												k					
HNO-Klinik			b ⁺⁺									k				o	p
Medizinische Klinik			b ⁺⁺	BH	c			f			i				n	o	p
Frauenklinik	a					e	e ⁺		h ⁺			k		m	n		
Sonstige			b ⁺⁺													o	p

Tabelle III zeigt, daß die isolierte Analyse der Antigene und die Aufstellung eines Antigenmosaiks auf Grund der signifikanten Differenzen eine deutliche Abgrenzung von Stämmen einzelner Kliniken ermöglichte. Daher wurde versucht, mit dieser Methode Beziehungen zu den Krankheitsbildern nachzuweisen. Wie wir feststellen konnten, waren die Stämme aus dem Material von Kliniken, in denen meist Patienten mit typischen klassischen Staphylokokkenprozessen zur Behandlung bzw. Aufnahme kamen, wie z. B. der Chirurgischen, Kinder- und Frauenklinik, durch die Antigene e' sowie h⁺ und/bzw. oder h⁺⁺ oder die beiden letzten zusammen charakterisiert. Durch weitere Nachforschungen bestätigten sich unsere Vermutungen. Tatsächlich waren die genannten Antigene bei den Staphylokokken solcher Prozesse, nämlich Mastitiden, Infektionen des lymphatischen Systems, Panaritien, Furunkeln, Phlegmonen und Abszessen, zu finden. Eines bzw. beide Antigene erwiesen sich also auch typisch für Infektionen der Haut und des Parenchyms innerer Organe. Zu diesem Schluß kam man, wenn die zahlreichen Staphylokokkeninfektionen in drei größere Sammelgruppen zusammengefaßt wurden, zu denen die eben genannten sowie Infektionen der Schleimhäute gehörten. Von den Staphylokokken der erwähnten Kliniken unterschieden sich jene aus Einrichtungen, in denen Patienten zur Aufnahme bzw. Behandlung kamen, die unter keinen oder wesentlich seltener unter klassischen oder typischen Infektionen litten. Es waren dies die HNO- und Medizinische Klinik sowie sonstige Einrichtungen. Den Stämmen von jenen Kranken waren die Antigene b⁺⁺ und o gemeinsam.

Infektionen, deren Erreger diese Antigene aufwiesen, betrafen den Urogenital-, Darm- und Respirationstrakt, bzw. es waren Otitiden. Es handelte sich demnach, wie vermutet, nicht um typische Staphylokokkenerkrankungen. Das Antigen b⁺⁺ kam bei ihnen mit o gekoppelt vor, bzw. war letzteres allein

vertreten. Die Kombination erwies sich ferner für Erreger von Schleimhautinfektionen typisch.

Es ließen sich hiermit die Vermutungen bestätigen, nach denen eine Beziehung zwischen dem serologischen Aufbau der Staphylokokken und den Kliniken existierte, in welchen die Kranken wegen bestimmter Infektionen behandelt wurden bzw. zur Aufnahme kamen. Demgegenüber bestanden zwischen den Erregern von Patienten einiger Kliniken auch serologische Differenzen, welche durch den unterschiedlichen Antigenaufbau der Keime aus den entsprechenden Infektionen resultierten.

Diskussion

Bisher hat man die Serologie meist zur Klärung des epidemiologischen Zusammenhangs beim Auftreten von Erkrankungen während des Krankenhausaufenthaltes oder Lebensmittelvergiftungen herangezogen, wie den folgenden Beispielen zu entnehmen ist.

OEDING teilte 1952 mit [1], daß 90% aller Mastitisfälle in Ost- und Westnorwegen durch einen bestimmten serologischen Typ hervorgerufen wurden. 1954 beobachtete man im Malben-Hospital von Beer-Yaacov einen Anstieg der postoperativen Wundinfektionen auf 37%. Wie die Nachforschungen ergaben, hatte der Infektionsstamm die Seroformel a b c. Er wurde nur noch bei zwei Personen des Operationsteams gefunden [13].

Über eine Serie von 20 autoptisch gesicherten Staphylokokkenfällen, bei denen der gleiche Serotyp oft nachgewiesen werden konnte, berichteten KIKUTH und GRÜN [14]. Ein Jahr später sahen LAUTERMANN und GRÜN [15] postoperative Wundinfektionen bei sechs Patienten, die durch Staphylokokken mit der serologischen Formel b ausgelöst wurden. Derselbe Stamm fand sich in der Nase und dem Rachen sowie an den Händen eines Arztes.

Bei einer kleineren Epidemie postoperativer Wundinfektionen, die durch Staphylokokken mit der Formel a b c e f h i k l o verursacht waren, züchtete GRÜN [16] Keime mit denselben Eigenschaften von den Händen eines Arztes, der Operationen ohne Gummihandschuhe ausführte.

Als Quelle einer Lebensmittelvergiftung durch Staphylokokken der Formel a c i ermittelten LÖNS und GRÜN [17] zwei Personen, welche an der Zubereitung der Speisen beteiligt waren und die angeschuldigten Erreger beherbergten.

Einige Autoren führten serologische Typisierungen durch, die gewisse Abweichungen in Abhängigkeit von der Herkunft der Erreger aufdeckten. So wurde z. B. nachgewiesen, daß Stämme aus schweren pathologischen Prozessen serologisch von Staphylokokken aus Nase und Rachen differierten [18]. Anlässlich einer Mastitisepidemie in Norwegen gelang es OEDING [2], die iso-

lierten Mastitisstämme von anderen zu unterscheiden. VISCHER [19], dessen Methode sich von jener unterschied, fand bei gehäuften Erkrankungen von Mastitis Unterschiede in der prozentualen Typenverteilung zwischen Staphylokokken dieser Infektionen und solchen aus anderen Quellen.

Von besonderem Interesse sind die serologischen Studien von UJVÁRY *et al.* [20] sowie ANGYAL [21], welche *S. aureus* Stämme aus eitrigen Prozessen und Blutkulturen sowie Stuhlproben einer pädiatrischen Einrichtung typisierten und die Gruppenbestimmung nach dem Schema von OEDING und WILLIAMS [11] vornahmen. Ein Vergleich der Ergebnisse zeigt, daß die Eiterstämme öfter den Gruppen 1 und 4 angehörten, wogegen die fäkalen Staphylokokken häufiger inagglutinabel und der Gruppe 2 zuzuordnen waren. Derartige Differenzen im serologischen Aufbau von Stämmen existieren auch zwischen denen weiterer Krankheitsbilder. Wie wir nachweisen konnten, bestehen zwischen den Staphylokokken aus mehreren Diagnosegruppen vielfältige Abweichungen hinsichtlich der Zugehörigkeit zu Gruppen, Typen und des Antigenaufbaus [22]. Wie wir gesehen haben, traf das auch für die Kliniken zu.

Für die Unterstützung bei der Durchführung der Berechnungen sei Herrn Dipl.-Mathem. PEUKER vom Rechenzentrum der Friedrich-Schiller-Universität gedankt.

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OBITUARY



Károly Rauss

Professor Károly Rauss, formerly principal of the Institute of Microbiology, University Medical School, Pécs, died on February 27, 1976. He was born in 1905. He received his medical education at the University Medical School, Budapest, graduating in 1929. He began to study microbiology in 1928 as a student at the institute of H. Preisz. Soon after graduation he entered the National Institute of Public Health, Budapest, where he became head of the Department of Bacteriology in 1939. In 1946 he was appointed Director of the Institute of Public Health, University Medical School, Pécs, then in 1951 Director of the newly established Institute of Microbiology, where he worked until his retirement in 1975.

During nearly 50 years of research he wrote 134 papers and published 3 monographs. He directed his energies towards solving many problems of antigenic structure (*Salmonella*, *Shigella*, *Morganella morganii*, etc.) and pathogenesis (dysentery, salmonellosis, etc.) and applied successfully the results of basic research to practical vaccination.

He was well known in his own country and abroad. He was a member of the International Committee on Systematic Bacteriology of the International Association of Microbiological Societies and of many Hungarian and foreign societies and committees. From the beginning he was an active member of the Editorial Board of *Acta Microbiologica Hungarica*.

During 30 years of professorship he was a very competent teacher. His high standards, his enthusiasm, his inventive and self-critical mind were an inspiration to his students and staff. His reputation for fairness and modesty helped him make friends all over the world. With the passing of Professor Károly Rauss, Hungarian and international microbiology has lost an outstanding scientist. He will be remembered by his friends, colleagues and pupils as a wise investigator, tireless teacher and devoted chief.

Iván Kétyi



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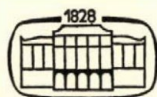
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STABILITY OF MAMMALIAN TUBERCULIN PPD

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(Received January 20, 1975)

Summary. Six lots of PPD prepared in 1937 met the requirements when tested in guinea pig potency assay in 1974. Reading of the skin reactions at 48 hr gave more consistent results than the 24 hr reading. Data were evaluated by analysis of variance and by a quick method developed for the estimation of identity of the standard and the test preparation. Although less sensitive than the analysis of variance, the quick method has been found suitable for estimation of the potency of tuberculin preparations.

Six lots of PPD prepared in 1937 were tested for potency in 1974. The relative potency values of the test preparations compared to the standard were calculated by analysis of variance and by a quick method developed by the present authors [1, 2].

Materials and methods

Tuberculin standard. Hungarian Reference Purified Protein Derivative (HR-PPD) Lot No. 5 was used [1]. HR-PPD contains 160 000 IU per ampoule.

PPD preparations. PPD lots H₁S, H₅S, H₁K, H₅K, H₁₀ and T were prepared according to SEIBERT [3] in 1937. The culture filtrates were precipitated with trichloroacetic acid (TCA) at a final concentration of 10%. The precipitate was washed with 10% TCA until the supernatant was colourless, then washed with ether and dried. The dry material was ground in a ball mill and stored at room temperature.

Biological assay. The potency tests were performed according to the Requirements for Tuberculin of the WHO [4]. Random-bred white guinea pigs of both sexes weighing 400–450 g, were immunized by the intramuscular injection of 2 mg dried *Mycobacterium tuberculosis* suspended in 1 ml of sterile liquid paraffin. As calculated on the basis of their tuberculoprotein content, the PPD preparations to be tested were assumed to contain 100 000 IU in 2.8 mg of PPD. Potency assays were done with antigen doses of 20 and 100 IU in 0.1 ml. Reactions were read 24 and 48 hr after inoculation.

Statistical analysis. Results were evaluated by analysis of variance [5] and our quick method [1, 2].

Results and discussion

Results of the analysis of variance are presented in Tables I–VI. Using 2 + 2 dose assay only three sources of variation (preparation, regression and parallelism) can be estimated. It can be seen that out of the 6 preparations to be tested H₅S (24 hr reading), H₁S and T (48 hr reading) differed significantly from the reference preparation. Regression lines were parallel.

Table I
Analysis of variance of preparation H₁S

Adjustment for mean	24 hr reading					48 hr reading				
	d.f.	2992.666 Sum of squares	Mean square	F	P	d.f.	2109.375 Sum of squares	Mean square	F	P
Preparations	1	0.666	0.666	0.381	> 0.1	1	3.375	3.375	5.551	< 0.05
Regression	1	48.166	48.166	27.523	< 0.005	1	70.041	70.041	115.199	< 0.005
Parallelism	1	1.500	1.500	0.857	> 0.1	1	0.041	0.041	0.067	> 0.1
Between doses	3	50.333				3	73.458			
Error (within doses)	20	35.000	1.750			20	12.166	0.608		
Total	23	85.333				23	85.625			

Table II
Analysis of variance of preparation H₁K

Adjustment for mean	24 hr reading					48 hr reading				
	d.f.	3060.041 Sum of squares	Mean squares	F	P	d.f.	2090.666 Sum of squares	Mean squares	F	P
Preparations	1	2.041	2.041	1.814	> 0.1	1	2.666	2.666	4.443	≤ 0.05
Regression	1	63.345	63.345	56.333	< 0.005	1	96.000	96.000	160.000	< 0.005
Parallelism	1	5.041	5.041	4.480	< 0.05	1	2.666	2.666	4.443	≤ 0.05
Between doses	3	70.458				3	101.333			
Error (within doses)	20	22.500	1.125			20	12.000	0.600		
Total	23	92.958				23	113.333			

Table III
Analysis of variance of preparation H₅S

Adjustment for mean	24 hr reading					48 hr reading				
	d.f.	3174.000	Mean square	F	P	d.f.	2072.041	Mean square	F	P
Nature of variation		Sum of squares					Sum of squares			
Preparations	1	6.000	6.000	6.211	< 0.025	1	2.041	2.041	4.296	> 0.05
Regression	1	54.000	54.000	55.901	< 0.005	1	84.375	84.375	177.631	< 0.005
Parallelism	1	2.666	2.666	2.759	> 0.1	1	1.041	1.041	2.191	> 0.1
Between doses	3	62.666				3	87.458			
Error (within doses)	20	19.333	0.966			20	9.500	0.475		
Total	23	82.000				23	96.958			

Table IV
Analysis of variance of preparation H₅K

Adjustment for mean	24 hr reading					48 hr reading				
	d.f.	2860.166	Mean square	F	P	d.f.	2016.666	Mean square	F	P
Nature of variation		Sum of squares					Sum of squares			
Preparations	1	0.166	0.166	0.122	> 0.1	1	0.666	0.666	2.503	> 0.1
Regression	1	54.000	54.000	40.000	< 0.005	1	80.666	80.666	303.255	< 0.005
Parallelism	1	2.666	2.666	1.974	> 0.1	1	0.666	0.666	2.503	> 0.1
Between doses	3	56.833				3	82.000			
Error (within doses)	20	27.000	1.350			20	5.333	0.266		
Total	23	83.833				23	87.333			

Table V
Analysis of variance of preparation H₁₀

Adjustment for mean	24 hr reading					48 hr reading				
	d.f.	2904.000	Mean square	F	P	d.f.	1908.166	Mean square	F	P
Nature of variation		Sum of squares					Sum of squares			
Preparations	1	0.000	0.000	0.000	> 0.1	1	0.166	0.166	0.269	> 0.1
Regression	1	60.166	60.166	35.749	< 0.005	1	80.666	80.666	130.951	< 0.005
Parallelism	1	4.166	4.166	2.475	> 0.1	1	0.666	0.666	1.081	> 0.1
Between doses	3	64.333				3	81.500			
Error (within doses)	20	33.666	1.683			20	12.333	0.616		
Total	23	98.000				23	93.833			

Table VI
Analysis of variance of preparation T

Adjustment for mean	24 hr reading					48 hr reading				
	d.f.	2816.666	Mean square	F	P	d.f.	1768.166	Mean square	F	P
Nature of variation		Sum of squares					Sum of squares			
Preparations	1	0.666	0.666	0.370	> 0.1	1	4.166	4.166	5.951	< 0.025
Regression	1	54.000	54.000	30.000	< 0.005	1	88.166	88.166	125.951	< 0.005
Parallelism	1	2.666	2.666	1.481	> 0.1	1	1.500	1.500	2.142	> 0.1
Between doses	3	57.333				3	93.833			
Error (within doses)	20	36.000	1.800			20	14.000	0.700		
Total	23	93.333				23	107.833			

Regression coefficients of preparations H_1S , H_1K , H_5S , H_5K , H_{10} and T at 24 hr reading were 2.83; 3.25; 3.00; 3.00; 3.17 and 3.00, respectively. Regression coefficients of the same preparations at 48 hr reading were 3.42; 4.00; 3.75; 3.67; 3.67 and 3.83, respectively.

From the analysis of variance the relative potency of the test preparations compared to the HR-PPD and their 95% fiducial limits were estimated. In addition, identity of the test and the reference preparations was estimated by the quick method. Data of Table VII show a positive correlation between the results of analysis of variance and those of the quick method. The quick method showed the test preparation to be identical with the reference preparation if its potency was $\pm 20\%$ within the limits of the standard. Accordingly, the 6 lots were found identical with the reference preparation as estimated by the quick method.

When considering the results of the variance analysis of both the 24 hr and the 48 hr data, the test preparations were partly identical with the reference preparation but in some cases differences were near to the limit of significance. Relative potency values were within the limits accepted by the WHO. The relative potency of H_5S (24 hr reading) was, however, higher. In this case, it has to be taken into account that the time of the reading is an important parameter in the potency assay of tuberculin. In Table VII it is seen that in the case of the 48 hr reading the fiducial limits of the relative potency values were acceptable in all the tests. At the 24 hr reading, however, all but one of the 95% fiducial limits exceeded the 75–130% range.

The tuberculin assay method used in 1937 differed from that used at present. Therefore the potency values found immediately after production cannot be compared with those found in 1974. The fact, however, that 6 lots of PPD prepared in 1937 have met the requirements in 1974 may be regarded as an indication of the stability of PPD.

Table VII

Potency of different preparations estimated by the quick method and by analysis of variance

Preparations	Time of reading, hr	Quick method,	Analysis of variance	
		per cent	RP	Fiducial limits
H ₁ S	24	102	1.100	0.769 — 1.635 (69.9% — 148.6%)
	48	108	1.200	1.023 — 1.418 (85.3% — 118.2%)
H ₁ K	24	106	1.160	0.923 — 1.393 (79.6% — 120.1%)
	48	107	1.140	0.986 — 1.337 (86.5% — 117.3%)
H ₅ S	24	109	1.310	1.045 — 1.712 (79.8% — 130.7%)
	48	107	1.130	1.093 — 1.184 (96.7% — 104.8%)
H ₅ K	24	97	0.958	0.719 — 1.262 (75.1% — 131.7%)
	48	104	1.070	0.978 — 1.188 (91.4% — 111.0%)
H ₁₀	24	99	1.003	0.762 — 1.389 (75.9% — 138.5%)
	48	99	0.976	0.840 — 1.160 (86.1% — 118.9%)
T	24	97	0.915	0.647 — 1.258 (70.7% — 137.5%)
	48	93	0.840	0.714 — 0.976 (85.0% — 116.2%)

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DECREASED CELLULAR IMMUNE RESPONSE OF GERM-FREE MICE

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(Received May 26, 1975)

Summary. Cellular immune response to intracerebral lymphocytic choriomeningitis infection was studied in mice belonging to an identical strain but different in breeding conditions. In consequence of the cellular immune reaction on the leptomeninx, lymphocytic choriomeningitis developed and caused death in 100% of conventionally bred mice, whereas 80% of germ-free and 15% of mouse-pathogen-free mice failed to display lymphocytic infiltration of the leptomeninx and survived the infection as chronic virus carriers. This finding pointed to a deficient cellular immune response of germ-free and mouse-pathogen-free mice. The under-development of the lymphoid system due to the antigen-poor breeding conditions might be responsible for the deficiency.

Antigenic stimuli being absent during the breeding of germ-free animals, their lymphoid system is underdeveloped [1–8]. In the present studies the course of intracerebral lymphocytic choriomeningitis (LCM) virus infection was studied in germ-free mice to obtain information on their cellular immune response, this being the factor determining the prognosis. Our preliminary observations on the course of LCM virus infection in germ-free animals have been published earlier [9].

Materials and methods

Animals. Twenty CD-1 germ-free mice, 20 CD-1 mouse-pathogen-free COBS (Cesarean Originated Barrier Sustained) mice (Charles River Breeding Laboratories Inc., Wilmington, Mass. USA) and 20 CD-1 conventional 6-week old male mice were used. The germ-free condition was maintained until the LCM virus infection or till the sacrifice of the animals.

Lymphocytic choriomeningitis virus. The WE strain was maintained in serial mouse-passages and stored at -70°C .

Reisolation of virus. Blood samples taken from the surviving mice by orbital puncture and the 1:10 dilution of their brain suspension was injected intracerebrally into mice. Recovery of the virus was established on the basis of the characteristic neurological symptoms.

Study of the lymphoid system. Absolute lymphocyte count was determined individually in blood taken under standardized conditions from the caudal vein. After sacrifice, the relative lymphoid organ weights were determined ($\frac{\text{organ weight mg}}{\text{body weight g}} = \text{relative organ weight}$).

Histology. Brain, spleen and thymus were removed after death, fixed in formalin and stained with haematoxylin-eosin.

Results

A pretitrated 100 LD₅₀ dose of LCM virus was injected intracerebrally in germ-free (Germ-free-LCM group), COBS (COBS-LCM group) and conventional (Conv-LCM group) mice. Neurological symptoms characteristic of LCM virus infection were observed and the occurrence of death was registered. Animals surviving for 21 days were sacrificed and their brain and blood were subjected to virus isolation experiments. Simultaneously with virus infection the lymphoid system was studied in germ-free, COBS and conventional mice.

The point of time of death in the virus infected groups is presented in Fig. 1.

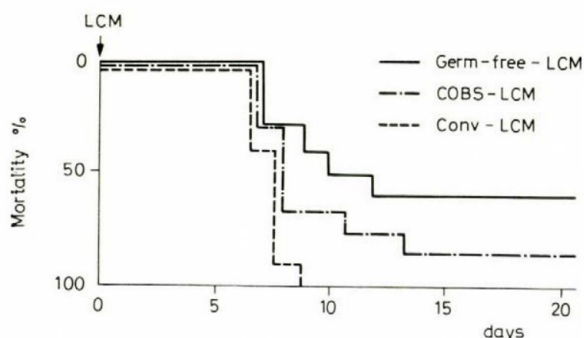


Fig. 1. Point of time of death in LCM virus infected mouse groups

All the animals of the Conv-LCM group developed neurological symptoms characteristic of lymphocytic choriomeningitis and died 7 to 9 days after the infection. In the Germ-free-LCM group only 20% of the animals developed symptoms of choriomeningitis and died simultaneously with the animals of the Conv-LCM group. No neurological symptoms were observed in 80% of the animals of the Germ-free-LCM group and 40% survived the 21st day post-infection. In the COBS-LCM group 85% of the animals developed symptoms of lymphocytic choriomeningitis and died between the 7th and 13th day. Fifteen percent failed to show neurological symptoms and were alive on the 21st day. Every brain and blood sample of the animals sacrificed on the 21st day contained LCM virus.

Lymphocytic infiltration of the leptomeninx was revealed by histology only in animals displaying neurological symptoms, thus in each animal of the Conv-LCM group, in 85% of the COBS-LCM group and in 20% of the Germ-free-LCM group. Lymphocytic infiltration of the leptomeninx was absent in animals of the Germ-free-LCM group which died without neurological symptoms or in those surviving the infection in the chronic virus carrier state.

Fig. 2 demonstrates the data for the lymphoid system of mice bred under different conditions.

Comparison of the absolute lymphocyte counts and mean relative lymphoid organ weights in the individual groups indicated that the lymphoid system of germ-free and COBS mice was underdeveloped as compared to that of conventional mice.

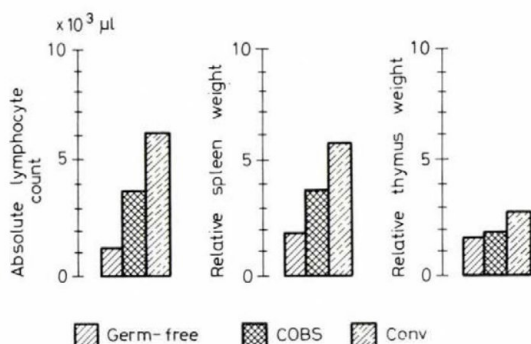


Fig. 2. Mean absolute lymphocyte count, relative spleen weight and relative thymus weight in germ-free, COBS and conventional groups of mice

Significant differences were revealed by histology of the lymphoid organs in mice bred under different conditions. The cortical substance of germ-free mice's thymus was cytopenic and contained practically no lymphoid cells. The spleen of conventional mice was normal in structure, with numerous germinative centres, whereas the spleen of germ-free mice was cytopenic and the germinative centres were absent. In COBS mice the thymus cortex was rather cytopenic and the spleen was similar as in the conventional mice.

Discussion

The course of LCM in germ-free and COBS mice differed from that observed in conventional animals, showing similarities to the course of neonatal infections and to those observed in adult mice thymectomized in newborn age [10-14]. The condition was characterized by a deficient cellular immune response due to underdevelopment of the thymus-dependent lymphoid system. Survival in the germ-free and COBS groups as well as the histological findings also indicated a deficient cellular type immune response. A direct correlation was found between the cellular immune response and the development of the lymphoid system. The latter appears to be correlated with the antigenic stress affecting the animals during their breeding. The lymphoid system was least developed in the germ-free animals and in COBS mice it was

also weaker than in conventional mice which had received the usual high number of antigenic stimuli.

On the basis of these findings, underdevelopment of the lymphoid system is considered responsible for the deficient cellular immune response of germ-free and COBS mice.

The results presented are in agreement with the findings of MIYAKAWA [15] who observed a decreased and retarded hypersensitivity reaction to mycobacteria in germ-free mice as compared with SPF mice. According to other data, *Mycobacterium bovis* infection runs a different course in germ-free and in SPF mice in that the former display a lower resistance. For this, the lack in germ-free mice of the microbial flora was made responsible [16].

Thus, besides the well-known causes of the deficient immune response, the lack of antigenic stimuli might also be responsible for the phenomenon, and that the infective diseases in adult animals bred under antigen-poor conditions differ from the classical pattern both clinically and histologically. At the same time it shows similarities to that observed in animals with deficient immune system.

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STUDIES ON THE LYMPHOID SYSTEM OF MICE WITH LETHAL ACUTE TOXOPLASMOSIS

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Summary. Acute toxoplasmosis was induced in CFLP mice by infecting them intraperitoneally with the 25×10^3 multiplicity of the virulent RH strain of *Toxoplasma gondii*. The lymphoid system of mice succumbing to acute toxoplasmosis showed characteristic changes. Significant spleen hypertrophy (spleen index: 1.76), severe thymus atrophy (thymus index: 0.27) and a striking decrease of the lymphocyte count in blood (86%) was found as compared with the uninfected controls.

Few experimental data are available concerning the immunosuppressive effect of parasitic infections. Observations have been reported in recent years on the immunosuppressive effect of trypanosomae and plasmodiae [1–3] as well as of trichinellae [4]. The immunosuppressive effect in mice of experimental toxoplasmosis has also been demonstrated [5, 6]. The decrease of immune function might be connected with the morphological changes of the lymphoid system, mainly of the thymus. Transient loss of thymus weight and hypertrophy of the spleen were considered characteristic of chronic toxoplasmosis of mice. Histological examinations showed that the thymus cortex was poor in lymphocytes. *Toxoplasma* infection appeared to influence the morphology and the function of the lymphoid system, but these correlations need further elucidation.

The above observations were made on mice with chronic toxoplasmosis induced by neonatal infection with a toxoplasma strain of low virulence.

We have therefore studied the lymphoid system of mice at the time of death from acute toxoplasmosis induced with the virulent RH strain of *Toxoplasma gondii*.

Materials and methods

Animals. Sixty, 6-week old CFLP mice of both sexes were used.

Toxoplasma strain. Standard international *Toxoplasma gondii* RH virulent strain was used. The strain was maintained by serial mouse-passages in our Institute. Intraperitoneal injection was applied once weekly with a toxoplasma suspension prepared from the abdominal exudate of mice showing symptoms of acute toxoplasmosis. The exudate was subjected to bacteriological sterility examination for 24 hr.

Study of the lymphoid system. The absolute lymphocyte count was determined under standardized conditions in blood samples obtained from the caudal vein. The ration of spleen

and thymus weight to body weight (relative organ weight) was determined at the time of death. On the basis of the relative lymphoid organ weights, the spleen and thymus indexes were determined in the individual groups.

Results and discussion

Fourty mice were infected intraperitoneally with the 25×10^3 multiplicity of toxoplasma. In earlier experiments this amount caused fatal acute toxoplasmosis in mice 6 to 9 days after infection. Each of the infected mice developed acute toxoplasmosis. Absolute lymphocyte count was determined in 20 infected and 20 control animals 7 days after infection. In order to determine the relative lymphoid organ weights, the animals were weighed and subsequently sacrificed. Spleen and thymus were removed and weighed. The remaining 20 animals died 7 to 8 days after infection and their relative lymphoid organ weights were determined as well. Data obtained on the lymphoid system of spontaneously dying (T_1 group), sacrificed (T_2 group), and control (C group) animals are presented in Fig. 1.

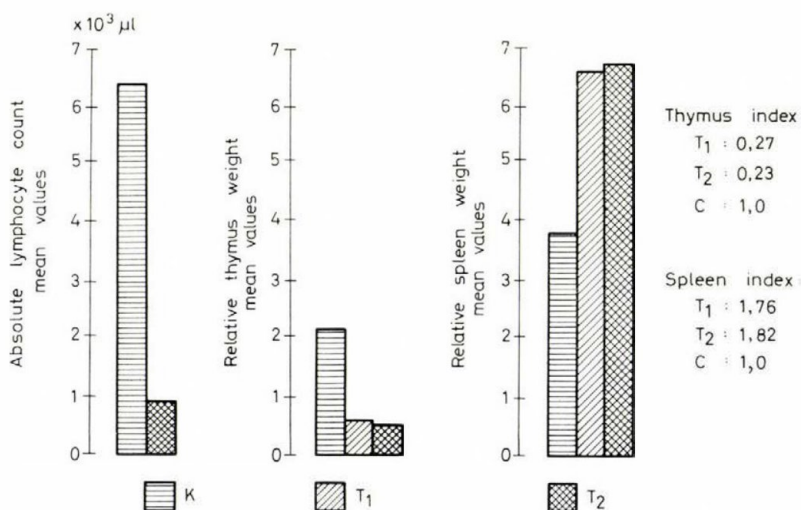


Fig. 1. Data characterizing the lymphoid system of the mouse groups

The findings reveal striking differences in the lymphoid system of mice with lethal acute toxoplasmosis (T_1 and T_2 groups) against controls (C group).

A low absolute lymphocyte count was found on the expected day of death, the decrease being 86% as compared to the controls. In addition, severe thymus atrophy occurred with a marked spleen hypertrophy, the degree of thymus atrophy and of spleen hypertrophy showed no difference between the sacrificed and the spontaneously dying infected animals.

Comparison of the present results with data on the lymphoid system of animals suffering from chronic toxoplasmosis [6] revealed that the effect on the lymphoid system was different in acute and chronic toxoplasma infection.

While a severe lymphopenia was characteristic of fatal acute toxoplasmosis no such phenomenon was detected in chronic toxoplasmosis.

The simultaneous existence of thymus atrophy and spleen hypertrophy was described also in chronic toxoplasmosis. However, while in chronic toxoplasmosis the thymus atrophy was transitory, in acute toxoplasmosis it appeared to be irreversible and the animals died with severe thymus atrophy. The same grave atrophy was observed in the sacrificed animals.

Comparison of the effects of chronic and acute toxoplasmosis showed that while the change of the thymus depended on the outcome of the infection, enlargement of the spleen was independent of the course of infection. Spleen hypertrophy appears to develop and exist always when toxoplasma is permanently present in the mice.

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ROLE OF C1 IN THE COMPLEMENT ACTIVATING EFFECT OF PSEUDOMONAS AERUGINOSA LIPOPOLYSACCHARIDE PREPARATIONS

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Summary. The complement consumption of endotoxin preparations extracted by the trichloroacetic acid or phenol-water method from different *Pseudomonas aeruginosa* strains was measured in normal human and guinea pig serum and in serum chelated with Mg^{2+} -EGTA. In the chelated serum, which was essentially Ca^{2+} -free, the first component of complement (C1) could not exert its function. All preparations tested consumed considerably less complement activity in chelated than in normal serum. The proportion of CH_{50} units fixed in Mg^{2+} -EGTA and in normal serum was always higher in the trichloroacetic acid extract than in the phenol-water extract of the same strain. The part of LPS molecule that was able to activate the complement system in Ca^{2+} -free serum was partially separated from the C1-requiring part by the combination of different extraction methods. The results suggest that on the LPS molecules two different sites are responsible for the complement activating effect through the classic and the alternative pathways.

Lipopolysaccharides isolated from the cell wall of Gram-negative bacteria are known as the most efficient activators of the complement system. 1. In the sera of animals immunized with Gram-negative bacteria LPS combined with the antibodies initiates the classic pathway of complement activation. 2. Erythrocytes coated with LPS can also be lysed by normal serum. The reaction requires the participation of a non-haemagglutinating γ_2 antibody and the classic complement pathway [1]. 3. LPS can directly consume the first component of complement [2]. 4. LPS activates the complement system via the alternative pathway, too [3].

Recently GIGLI *et al.* [4] reported on the dependence of complement activation through the alternative pathway induced by zymosan on the early acting components of complement. FRANK and MAY [5] described a C1 bypass activator pathway which requires only C1 from the early components. These findings have prompted us to study the role of the first component of complement (C1) in the complement activating effect of bacterial lipopolysaccharides. Mainly the method of FINE *et al.* [6] was used, but instead of ethylene glycol tetra-acetic acid (EGTA), Mg^{2+} -EGTA was used for the elimination of Ca^{2+} ions necessary for the functioning of the C1 macromolecule. LPS preparations isolated from different *Pseudomonas aeruginosa* strains by various methods, were included in the study. Most of the experiments were performed simultaneously in human and guinea pig serum.

Materials and methods

Diluents were prepared as described previously [7].

Sera. Pooled human serum from 15–25 subjects and pooled guinea pig serum from 4–6 animals were stored at -30°C .

Bacterial strains. *Pseudomonas aeruginosa* type strains of LÁNYI's [8] antigenic schema were used.

LPS preparations. *Trichloroacetic acid (TCA) extracts* were prepared by the method of BOIVIN *et al.* [9] by suspending 5 g of dry bacteria in 50 ml of ice cold TCA 10% w/v. The suspension was left to stand in the refrigerator at $4^{\circ}\text{--}6^{\circ}\text{C}$ for 24 hr and was then centrifuged. After a second extraction of the deposit, the supernatants were pooled, dialysed and freeze-dried.

Phenol-water extracts from bacteria were prepared and purified by the method of WESTPHAL and JANN [10] as described by ÁDÁM *et al.* [11].

Phenol-water extracts from TCA extracts were prepared by dissolving 150 mg of the latter in 30 ml of 45% phenol at 68°C [10]. To remove the phenol after separating the water and phenol phases, both the water and phenol layers were dialysed and then freeze-dried. These extracts were not subjected to subsequent ultracentrifugation. The yield of water phase and phenol phase extracts from TCA was 50.7 mg and 15.8 mg, respectively.

Measurement of complement consumption in human and guinea pig serum was performed by mixing 0.9 ml of undiluted serum with 0.1 ml of saline (controls) or 0.1 ml chelating agents (EGTA or EDTA, final serum concentration 10 mM). One tenth ml of the mixture was incubated with 0.1 ml of the different dilutions of LPS preparations at 37°C for 60 min. Then the chelated sera were recalcified with the corresponding volume of 100 mM CaCl_2 and their haemolytic complement activity was determined by the standard method [12]. Complement activity consumed by the LPS preparations was expressed as the difference in CH_{50} units measured in the control tubes (containing only normal or chelated serum) and in tubes containing the different doses of LPS preparations.

Preparation of purified complement components. Partially purified Cl_{hu} was prepared as described by TAMURA and NELSON [13]. Guinea pig C2 was obtained in partially purified form according to the method of NELSON *et al.* [14].

Preparation of sheep erythrocyte-antibody-complexes. EAC_{hu} was prepared as described by BORSOS and RAPP [15], $\text{EAC}_{\text{hu}}^4\text{hu}^2\text{gp}$ on the basis of the method of TOWNES and STEWART [16].

Preparation of Cl-depleted human serum. Human serum was depleted of Cl by low ionic strength precipitation [14]. This treatment decreased the concentration of other proteins (e.g. IgM) in the serum, but only the depletion of Cl was complete. Depleted serum diluted 1 : 5 during preparation was made isotonic by adding a sufficient amount of 9% saline.

Measurement of C3–C9 activity in human serum. The titration was performed on the basis of the method of TOWNES and STEWART [16]. Five tenths ml of freshly prepared $\text{EAC}_{\text{hu}}^4\text{hu}^2\text{gp}$ (1×10^8 cell/ml) was incubated with 1.0 ml of different dilutions of human serum in 0.01 M EDTA buffer at 37°C for 60 min. Then 3.0 ml of ice-cold isotonic saline was added to each tube, the tubes were centrifuged and the optical density of the supernatants was measured by spectrophotometry at 412 nm. The 50% end point was calculated by a graphic method according to the von Krogh equation [12]. C3–C9 activity of serum was expressed in CH_{50} /ml units.

Measurement of "detoxification" of LPS preparations in Mg^{2+} -EGTA serum. One tenth ml of human or guinea pig serum containing 10 mM EGTA and 8 mM MgCl_2 was incubated with 62.5 μg of LPS preparation at 37°C for 0–60 min, then recalcified with 100 mM CaCl_2 and reincubated at 37°C for 60 min. After the second incubation the residual complement activity was determined.

Results

According to FINE [17] human and guinea pig sera chelated with EGTA at 10 mM final concentration contained a concentration of Mg^{2+} ions (10^{-6} M) which could support the activation of alternative pathway. These experiments were performed with a large dose, 2 mg/ml of zymosan. Therefore we have

measured the complement activity consumed by different doses (0.125–8 mg) of zymosan in normal human serum and in serum chelated with 10 mM EGTA (Table I).

Table I

*Complement activity consumed by different doses
of zymosan in normal human serum
and in human serum chelated with 10 mM EGTA*

Dose of zymosan, mg	Complement activity consumed in	
	normal human serum* (CH ₅₀)	serum chelated with EGTA* (CH ₅₀)
8	25.0	25.0
4	25.0	25.0
2	25.0	25.0
1	25.0	25.0
0.5	21.3	7.65
0.25	14.0	5.25
0.125	9.75	1.25

One half ml of human serum was incubated with the pellet of a corresponding volume of boiled zymosan suspension (5 mg/ml) at 37°C for 60 min, then the zymosan was eliminated by centrifugation and the residual complement activity was measured.

* Complement activity available: 25.0 CH₅₀.

Higher doses of zymosan (> 1 mg) consumed 100% of the available complement activity in both normal and EGTA chelated serum, but lower doses fixed considerably less complement in chelated than in normal human serum (e.g. 0.25 mg of zymosan fixed 56% of the available complement activity in normal serum, but only 21% in EGTA serum; 0.125 mg fixed 39 and 5%, respectively).

In the subsequent experiment the complement activity consumed by 0.5 mg of zymosan in human serum chelated with EGTA but supplemented with MgCl₂ at different concentrations was measured. Normal and EDTA treated sera served as controls (Table II).

One half mg zymosan consumed 75% of the available complement activity (30 CH₅₀) in normal serum, but only 24.2% in EGTA treated serum. In serum chelated with EDTA no significant complement fixation was observed. Increase of Mg²⁺ concentration of serum led to an increase of complement consumption and in serum supplemented with 7 to 9 mM of MgCl₂ the same CH₅₀ units were fixed by zymosan as in normal serum. Therefore serum chelated with 10 mM of EGTA and supplemented with 8 mM of MgCl₂ (MgCl₂+EGTA serum) was used in further experiments.

Table II

Complement activity consumed by 0.5 mg zymosan
in human serum chelated with 10 mM EGTA
and supplemented with $MgCl_2$ at different concentrations

Final concentration in serum, mM		Complement activity consumed by zymosan CH_{50}
EGTA —	$MgCl_2$ —	22.50
10	—	7.25
10	1	15.20
10	3	19.15
10	5	21.45
10	7	22.50
10	9	22.50
EDTA 10	—	3.50

One half ml of serum was incubated with zymosan at 37°C for 60 min, then the zymosan was eliminated by centrifugation and the residual complement activity of the serum was measured.

“Detoxification” of LPS preparations in Mg^{2+} -EGTA serum. FINE [17] found that isolated LPS preparations were “detoxified” (had lost their complement consuming capacity) in EGTA treated serum in 5 to 10 min, and pointed out that isolated endotoxin was not suitable for such studies. We have therefore investigated whether “detoxification” of the LPS preparations occurs in Mg^{2+} -EGTA serum. The experiment was performed essentially as described by FINE [17] except for using Mg^{2+} -EGTA instead of EGTA for the chelation of serum (Fig. 1).

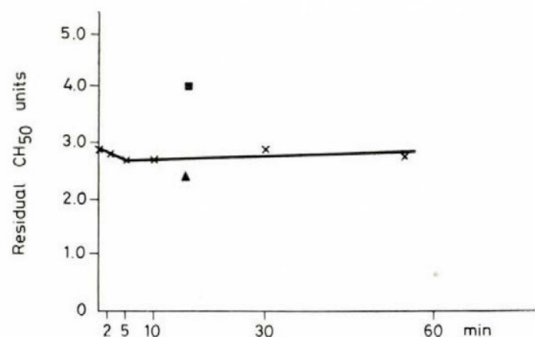


Fig. 1. Effect of preincubation of LPS in Mg^{2+} -EGTA serum on its complement-consuming ability in recalcified serum: 62.5 μ g of LPS were incubated in 0.1 ml of Mg^{2+} -EGTA serum at 37°C for 0–60 min, then recalcified and reincubated at 37°C for 60 min. After the second incubation, the residual complement activity was determined. $\times$ — $\times$: residual complement activity measured in tubes incubated for different times; $\blacksquare$ — $\blacksquare$ residual CH_{50} of Mg^{2+} -EGTA serum, incubated at 37°C for 60 min, recalcified and incubated at 37°C for another 60 min (control tube); $\blacktriangle$ — $\blacktriangle$ residual complement activity of normal serum incubated with 62.5 μ g of LPS at 37°C for 60 min

Results of one representative experiment are shown in Fig. 1 and in the subsequent Figure and Tables. In general, the experiments were repeated three times; there was no significant difference in the results.

As can be seen in Fig. 1, residual complement activity showed no significant differences when measured in tubes incubated in Mg^{2+} -EGTA serum at 37°C for 0 to 60 min (2.70–3.13 CH_{50}), and these values were considerably lower than the value (4.08 CH_{50}) measured in the control tube. After incubation of the same dose of endotoxin and normal human serum, residual complement activity was nearly the same (2.42 CH_{50}). Thus, preincubation of LPS in Mg^{2+} -EGTA serum did not markedly impair the ability of complement consumption in recalcified serum. Similar results were obtained in guinea pig serum.

Complement consumption of P. aeruginosa LPS preparations isolated by different methods in normal and Mg^{2+} -EGTA human and guinea pig sera. Complement consumption of LPS preparations extracted from the same *P. aeruginosa* strains by the trichloroacetic acid method and the phenol-water method was compared in normal and Mg^{2+} -EGTA sera. The results obtained with 62.5 μg of LPS are shown in Table III.

Table III

Complement consumption by trichloroacetic acid and phenol-water extracts prepared from P. aeruginosa strains in normal and Mg^{2+} -EGTA human serum (62.5 μg of preparation + 0.1 ml serum, 37°C, 60 min)

Designation of strain	Preparation	CH_{50} units consumed		
		in normal serum* (A)	in Mg^{2+} -EGTA serum* (B)	B/A
170016	Phenol-water	3.23	0.69	0.21
	TCA	2.50	1.98	0.79
170021	Phenol-water	2.84	1.33	0.47
	TCA	2.10	1.61	0.77
170019	Phenol-water	3.70	0.82	0.22
	TCA	2.04	0.81	0.40
170010	Phenol-water	2.34	1.00	0.43
	TCA	2.11	1.49	0.70
170014	Phenol-water	2.25	0.64	0.28
	TCA	1.94	1.29	0.67
Mean	Phenol-water	2.87	0.90	0.31
	TCA	2.34	1.44	0.62

* CH_{50} available: 5.0.

In normal serum TCA and phenol-water extracts consumed nearly equal complement activity, only the phenol-water extract of strain 170019 was considerably more active. In contrast, in Mg^{2+} -EGTA serum, except again 170019, TCA extracts consumed significantly more complement activity than did the phenol-water extracts. Neither of the preparations could, however, fix the same number of CH_{50} units in Mg^{2+} -EGTA serum as in normal serum.

The proportion of CH_{50} units consumed by the same preparation in Mg^{2+} -EGTA and in normal serum was considerably (1.5–3.19 times) higher in the case of all TCA extracts compared to the phenol-water extracts prepared from the same strain. When the experiments were performed in guinea pig serum or with lower or higher doses of LPS (Fig. 2), essentially the same results were obtained.

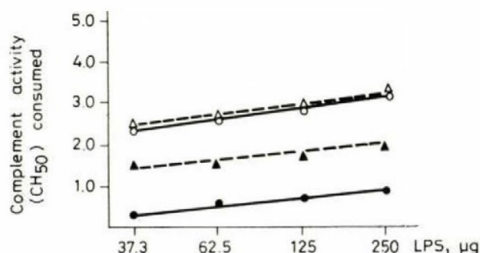


Fig. 2. Complement consumption by different doses of extracts prepared from *P. aeruginosa* strain 170016. Phenol-water extract ○—○ in normal human serum, ●—● in Mg^{2+} -EGTA serum. TCA extract △—△ in normal serum, ▲—▲ in Mg^{2+} -EGTA serum

Measurement of C3–C9 haemolytic activity consumed by LPS preparations in C1 depleted human serum. One and a half ml of human serum depleted of C1 by low ionic strength precipitation and 1.5 ml of normal human serum diluted 1 : 5 with saline was mixed with 0.1 ml of two different doses of LPS and incubated at 37°C for 60 min. Then the contents of each tube were further diluted with 0.01 M EDTA buffer and the residual C3–C9 activity was determined (Table IV).

In the normal serum, the two different doses of LPS preparations consumed 50 and 44% of the C3–C9 activity but in the C1-depleted serum there was only 14 and 8% C3–C9 consumption.

Partial separation of the part of LPS molecule responsible for complement activation in Ca^{2+} free serum. Phenol-water extract was prepared according to WESTPHAL and JANN [10] from a TCA extract (strain 170016) which showed

Table IV

*Consumption of late-acting complement components by phenol water extract of P. aeruginosa 170016 in normal human serum and in human serum depleted of C1 by low ionic strength precipitation**

Serum	250 µg LPS		125 µg LPS	
	C3-C9 activity consumed			
	CH ₅₀	in percentage of CH ₅₀ available*	CH ₅₀	in percentage of CH ₅₀ available**
Normal serum diluted 1 : 5	10.50	50	9.20	44
Serum depleted of C1; final dilution 1 : 5	1.65	12.1	0.88	8

* 1.5 ml serum incubated with 0.1 ml of LPS preparation at 37°C for 60 min.

** C3-C9 activity available in normal serum: 21.0 CH₅₀, in C1-depleted serum: 13.65 CH₅₀.

a comparatively strong complement consumption in Mg²⁺-EGTA serum. Complement consumption in normal and Mg²⁺-EGTA serum of both water and phenol phases is shown in Table V.

Table V

Complement consumption in normal and Mg²⁺-EGTA human serum by TCA extract and by water and phenol phases of purified TCA extract of P. aeruginosa 170016

Preparation	CH ₅₀ units consumed		
	in normal serum* (A)	in Mg ²⁺ -EGTA serum* (B)	B/A
TCA extract	2.64	1.55	0.58
Phenol-water extract prepared from TCA extract			
water phase	1.28	1.34	>1.0
phenol phase	4.50	0.82	0.18

* CH₅₀ available: 5.0.

The CH₅₀ units fixed by the original preparation in Mg²⁺-EGTA serum amounted to about 60% of the units fixed in normal serum. The water phase of the phenol-water extract prepared from the TCA extract could, however, fix complement to the same extent both in normal and in Mg²⁺-EGTA serum. In contrast, the phenol phase of the extract induced a strong complement consumption in normal serum, in Mg²⁺-EGTA serum, however, relatively few (19%) CH₅₀ units were fixed.

Discussion

Complement consumption of LPS preparations extracted from *P. aeruginosa* strains was studied in Ca^{2+} free serum in which the first component of complement (C1) could not exert its function. In comparison, complement consumption of the same preparation was measured in normal human and guinea pig serum. The method used was originally recommended by FINE *et al.* [6] for the distinction of materials acting on the complement system through the classic or alternative pathways. The original method was modified using Mg^{2+} -EGTA instead of EGTA for the chelation of serum. Serum chelated with EGTA in a final concentration of 10 mM contains 10^{-6} M Mg^{2+} ions which according to FINE *et al.* [6] can support the activation of the alternative pathway. These authors used a high dose of zymosan, and, as our experiments indicated, this concentration of Mg^{2+} ions is not enough to complete activation of the alternative pathway by lower doses (< 1 mg) of zymosan. If the system was, however, supplemented with MgCl_2 in a final concentration of 8 mM, even the smallest dose of zymosan used (0.25 mg) consumed the same CH_{50} units as it did in normal serum. Serum mixed with EGTA (10 mM) and MgCl_2 (8 mM) is essentially Ca^{2+} free and contains Mg^{2+} ions in approximately normal concentration (2 to 3 mM) [18]. Application of this modified method had further advantage. In human or guinea pig serum chelated with Mg^{2+} -EGTA the endotoxin was not "detoxified": LPS incubated in Mg^{2+} -EGTA serum did not lose its ability to consume complement activity in recalcified serum. Incubation of LPS in serum chelated with EGTA considerably impaired, however, its complement consuming capacity [17]. It seems that Mg^{2+} ions have a protective effect against "detoxification". These results are in agreement with the findings of DIERICH *et al.* [19].

The comparative study of *P. aeruginosa* LPS preparations extracted either by the trichloroacetic acid or by the phenol-water method in normal and Mg^{2+} -EGTA serum led to the following conclusions.

1. Neither of the LPS preparations studied (even in a very high dose: 2.5 mg/ml of serum) could activate the complement system to the same extent in Ca^{2+} free as in normal human or guinea pig serum. The proportion of the CH_{50} units consumed in normal and in Mg^{2+} -EGTA serum was nearly the same when using human or guinea pig serum. The conclusion is based on the study of 10 different *P. aeruginosa* LPS preparations. The same results were obtained with a *Serratia marcescens* TCA extract and with different *Escherichia coli* LPS preparations (unpublished data). Similar results were obtained by a different method (depletion of serum in C1 by low ionic strength precipitation). These findings can be explained by the simple fact that these LPS extracts activate both the classic and the alternative pathway and the propor-

tion of CH_{50} units fixed in Mg^{2+} -EGTA and normal serum shows the proportion of the ability of the preparations to activate the complement system via the two pathways. But analysing the results of experiments on the mechanism of complement activation induced by endotoxin, the possibility of a third pathway of action arose. Studies of GEWURZ *et al.* [20], GÖTZE and MÜLLER-EBERHARD [3] and FRANK *et al.* [21] clearly demonstrated that LPS efficiently activated the alternative pathway, i.e. activated components of the alternative pathway (properdin, B, D factor) and C3, and apparently failed to consume the early-acting components. On the other hand, other experiments suggested that the role of early-acting components cannot be excluded from the mechanism of complement activation induced by endotoxin. SNYDERMAN *et al.* [22] observed that the consumption of the late-acting components significantly decreased in serum in which C4 was inactivated. We obtained similar results in experiments on adrenalectomized rats [7]. GEWURZ *et al.* [23] demonstrated the decrease of C3-C9 consumption induced by LPS in congenitally C1 and C2 deficient sera. PHILLIPS *et al.* [1] showed that the lysis of erythrocytes coated with LPS is mediated through the classic pathway.

Taking these findings into account, it seemed reasonable to suppose that in the mechanism of complement activation induced by endotoxin the components of both classic and the alternative pathways may participate. C3b generated by the classic pathway may initiate the amplification cycle and so the alternative pathway, too, is activated. Experiments of MARCUS *et al.* [24] indicated, however, that C3-convertase (C42) was not formed on the surface of LPS particles. Thus it seems more probable that the early acting components (C1 and perhaps C4) could directly activate one of the components of alternative pathway. Further experiments are needed to support this hypothesis but recently a similar new cytotoxic mechanism called C1 bypass pathway requiring C1 and components of the alternative pathway has been described by FRANK and MAY [5]. On the basis of the available data, however, the activation of the classical pathway by *P. aeruginosa* endotoxins through the natural non-haemagglutinating antibodies [1] cannot be excluded.

2. Results of our experiments clearly showed that the majority of *P. aeruginosa* LPS preparations activated directly (without the participation of C1) the alternative pathway, although the number of CH_{50} units consumed by this type of activation had never reached the number of units consumed in normal serum.

The proportion of CH_{50} units fixed in Mg^{2+} -EGTA serum and in normal serum was always higher in the case of the TCA extract than of the phenol-water extract prepared from the same bacterial strain. Furthermore, we could partially separate the complement activating activities requiring and not requiring C1, by further purification of a TCA extract using the phenol-water

method. The aqueous phase of this extract consumed complement to the same extent in both Mg^{2+} -EGTA and normal serum but the phenol phase fixed considerably less CH_{50} units in Ca^{2+} free than in normal serum. These findings suggest that different parts of the LPS molecule are responsible for the complement activating effect utilizing the classic (or the C1 bypass) pathway and the alternative one.

It is known that in certain bacteria such as *Xanthomonas campestris* and *Citrobacter freundii*, LPS may be isolated from the phenol phase of the phenol-water extract [25]. In the case of *P. aeruginosa*, LPS may be separated from the aqueous and the phenol phase as well. *P. aeruginosa* phenol phase LPS sensitizes sheep erythrocytes similarly as does aqueous phase LPS. On hydrolysing the phenol phase LPS by acid, the fatty acids are precipitated and the sugar content of the polysaccharide moiety will be the same as that of the water phase LPS [26]. It is assumed that the difference in complement activating activity between water and phenol phase LPS is due to unclarified differences in the lipid moiety of LPS molecules in the two phases of the extract.

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R FACTORS DERIVED FROM SHIGELLA FLEXNERI STRAINS

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Summary. Of 2492 *Shigella flexneri* strains isolated from dysenteric patients in Hungary in the years 1972–1974, 767 (30.8%) were resistant to 1–5 antibiotics. Resistance was due to R factors in 79.2% of the strains. Fertility inhibition experiments with F specific phages showed R factors to be fi^+ in 12.9% and fi^- in 87.1%. Of the antibiotic markers, the chloramphenicol–tetracycline resistance determinant was the most common (46.4%). Chloramphenicol resistance determinants were carried also by fi^- R factors.

Multiresistant *Shigella flexneri* was first isolated from a dysentery outbreak by KITAMOTO *et al.* [1] in Japan in 1955. Genetic analysis revealed that multiple drug resistance was due to an extrachromosomal element which was then termed antibiotic resistance factor or R factor [2–4]. Resistance transfer was first demonstrated in Europe by DATTA [5] in a *Salmonella typhi-murium* strain that caused a protracted outbreak of hospital infection. Since that time extensive epidemiological studies have been performed throughout the world [6–11] showing that in numerous clinical situations R factors are the agents responsible for the resistance to antibiotics of *Enterobacteriaceae*. The occurrence of R^+ strains is not limited to hospital outbreaks; R factors are present in domestic animals [8], and fish [12], particularly when antibiotics are used as routine dietary additives. SMITH [13] reported that R^+ *Escherichia coli* strains derived from urban sewage were found in river water.

As the appearance and spread of strains containing R factors is a serious problem in epidemiology, medical treatment and public health, it seemed us desirable to obtain data on the occurrence of *S. flexneri* strains resistant to antibiotics and to determine the ratio of R factor carrying strains in Hungary.

Materials and methods

Strains. A total of 2492 *S. flexneri* strains originated from dysenteric patients in Hungary. As recipient strain *E. coli* K12 Hfr H $nal^r lac^+$ was used.

Phages. A phage-set containing 19 type phages was used for phage typing of the *S. flexneri* strains [14]. Male-specific phages: Ms_2 [15] and f_2 [16] were applied for determining fi character.

Culture media. Nutrient broth agar and eosin methylene blue agar containing streptomycin, chloramphenicol, tetracycline (30 μ g/ml) and/or nalidixic acid (50 μ g/ml) were used as selective media. Sensitivity was examined to streptomycin (Sm), chloramphenicol (Cm),

tetracycline (Tc), neomycin (Nm) and polymyxin B (Pm) by the disk method, using "Resistest" disks ("Human", Budapest) and Mueller-Hinton agar.

Crosses were carried out by the method of TSCHÄPE and RISCHE [17]. The 18 hr broth cultures of the donor and recipient strains were centrifuged, the sediments mixed (1 : 1) and seeded on agar plates. After incubation at 37°C for 5 hr the cultures were suspended in broth and the undiluted and diluted mixtures were plated on selective media and incubated at 37°C for 16 hr. The colonies were subcultured three times on agar plates containing the different antibiotics.

Determination of fi character. Each R factor was classified as fi^+ or fi^- on the basis of repression of F fimbria after transfer to *E. coli* K12 Hfr H. This was tested by examination of the Hfr R^+ culture for visible lysis on plating with phages Ms_2 and f_2 . According to MEYNELL and DATTA's report [18] this method is a reliable test for the fi character.

Results

Antibiotic sensitivity of S. flexneri strains. Out of the 2492 *S. flexneri* strains examined, 797 (30.8%) were resistant to 1–5 antibiotics. As shown in Fig. 1, the percentage of resistant strains was 26.8 in 1972, 30.1 in 1973, 35.8 in 1974, 35.8 in 1974.

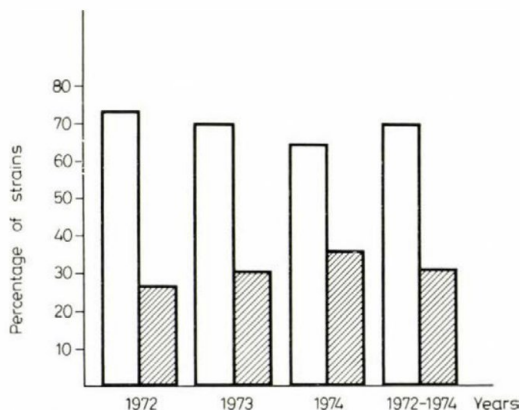


Fig. 1. Distribution of *S. flexneri* strains according to sensitivity to antibiotics. Open columns sensitive to antibiotics (Sm, Cm, Tc, Nm, Pm); shaded columns: resistant to 1 to 5 antibiotics

The order of frequency of serotypes of *S. flexneri* was 3a, 4a, 2a, 6, var. X. Strains resistant to antibiotics were the most common among the strains of serotype 6 (45.3%). The incidence of resistant strains of serotype 2a and 3a was 33.8% and 32.2%, respectively. The ratio of resistant strains of serotype 4a and var. X were nearly similar (23.3%, 27.7%).

Table I presents the distribution of *S. flexneri* strains according to antibiotic resistance. Though according to the summarized data of three years (1972–1974) the number of strains resistant to one, two and three antibiotics was nearly identical (242, 253, 235 strains, i.e. 31.6%, 33.0%, 30.6%), in the yearly analysis for 1972 and 1973 resistance to one and two antibiotics was frequent (69.2% and 74.7%), but to three and four antibiotics it was not so

Table I
*Distribution of S. flexneri strains according
 to antibiotic resistance in Hungary, 1972-1974*

Resistant to		No. of strains			Total
		1972	1973	1974	
1 antibiotic	Sm	12	29	28	69
	Cm	40	14	12	66
	Tc	18	51	31	100
	Nm	2	1	—	3
	Pm	—	—	4	4
2 antibiotics	Sm-Cm	4	3	26	33
	Sm-Tc	1	2	4	7
	Sm-Pm	—	1	—	1
	Sm-Nm	—	1	—	1
	Cm-Tc	80	92	28	200
	Tc-Pm	—	1	10	11
3 antibiotics	Sm-Cm-Tc	67	40	110	217
	Sm-Tc-Pm	—	3	—	3
	Sm-Cm-Nm	—	2	6	8
	Cm-Tc-Pm	—	3	4	7
4 antibiotics	Sm-Cm-Tc-Nm	3	14	15	32
	Sm-Cm-Tc-Pm	—	4	1	5
Total		227	261	279	767

common (30.8% and 25.3%). In 1974 the occurrence of strains resistant to one and two antibiotics was lower (51.3%) and strains resistant to three and four antibiotics became more frequent (48.7%).

Concerning the antibiotic resistance markers among the strains resistant to a single antibiotic in 1972 Cm, in 1972 and 1973 Tc and Sm resistance were the most frequent. The most common resistance-patterns were Cm-Tc in 1972 and 1973, and Sm-Cm-Tc in 1974. Resistance to neomycin and polymyxin was infrequent.

Occurrence of R factors. Our studies were aimed at determining the occurrence of R factor carrying *S. flexneri* strains in Hungary. All of the examined multiple resistant strains isolated in 1967 and 1968 possessed R factors [19]. The resistant strains tested in 1972-1974 proved to be R factor carriers in 79.2%.

Table II shows the distribution of R factors according to resistance determinants and *fi* character. Among the resistance determinants, Cm-Tc was the most frequent (46.4%) in the 420 R factors tested. The R factors carried Sm-Cm-Tc determinants in 16.4%, single Cm determinant in 11.9%. Difference was observed in the incidence of Cm and Tc determinants in the three years: in 1972 the Tc determinant was carried by only 5 and the Cm determinant by 26 R factors while in 1973 by 18 and 17 and in 1974 16 and 6 R factors, respectively.

The R factors carried by *S. flexneri* strains proved to be *fi*⁻ in 87.1% and *fi*⁺ in 12.9%.

Table II

Distribution of R factors according to resistance determinants and fi character in Hungary, 1972-1974

Resistance determinants		No. of R factors							
		1972		1973		1974		Total	
		<i>fi</i> ⁺	<i>fi</i> ⁻	<i>fi</i> ⁺	<i>fi</i> ⁻	<i>fi</i> ⁺	<i>fi</i> ⁻	<i>fi</i> ⁺	<i>fi</i> ⁻
Sm		—	4	2	2	—	17	2	23
Cm		2	24	6	12	—	6	8	42
Tc		—	5	4	13	—	16	4	34
Sm-Cm		—	8	—	2	2	9	2	19
Sm-Tc		—	—	1	3	—	1	1	4
Cm-Tc		17	68	13	73	—	24	30	165
Sm-Cm-Tc		—	31	4	9	2	23	6	63
Sm-Cm-Nm		—	—	—	—	—	2	—	2
Sm-Cm-Tc-Nm		—	—	—	—	1	14	1	14
Total	No.	19	140	30	114	5	112	54	366
	%	11.9	88.1	20.8	79.2	4.3	95.7	12.9	87.1

Discussion

S. flexneri strains were resistant to antibiotics in 30.8%. Of the 2492 strains examined, 19.8% were resistant to one and two, 10.9% to three and four antibiotics. In the years 1967-1968 3.5% of 1336 *S. flexneri* isolates were resistant to 3-6 antibiotics. These data show an increase in the frequency of multiple resistant strains.

KÉTYI and VERTÉNYI [20] showed the episomic nature of resistant *Shigella* in Hungary. As described by KOLTA [21] the incidence of *Shigella*

strains resistant to Cm and Tc increased from 17.1% to 25.5% and from 15.9% to 45.3%, in the periods 1959–1960 and 1961–1967, respectively. CSISZÁR and SZOLCSÁNYI [22] found 71.4% of *S. flexneri* to carry R factor.

According to the compiled annual reports of the Hungarian Public Health Laboratory Network, in the years 1972 and 1973, *S. flexneri* strains were sensitive to neomycin in 92.7% and 92.9%, to polymyxin B in 89.6% and 92.4%, to chloramphenicol in 72.4% and 76.7%, to streptomycin in 60.7% and 59.8%, and to tetracycline in 59.2% and 46.4%, respectively.

Though the ratio of resistant strains found by us is lower than that reported from other countries, the situation cannot be considered promising because the number of resistant strains may further increase in the future if antibiotic therapy will not be used purposefully and drugs used in human and animal therapy will not be separated.

In Japan, multiple-resistant shigellae occurred in 10–20% in 1955–1956. Their frequency increased to 80% by 1968 and to 92% by 1969. Among the examined 10 462 strains 17.0% were resistant to one, and 70.0% to four antibiotics [23]. *Shigella* strains now isolated in hospitals are multi-resistant in more than 50% all over the world [24].

Reports on the drug resistance of shigellae mostly fail to specify to which subgroup they belong. To interpret the data one has to consider which subgroup is frequent in the given country. Among the strains belonging to the *S. sonnei* subgroup the number of drug resistant strains is usually higher than in those belonging to the *S. flexneri* subgroup.

In Washington [25] the percentage of *S. sonnei* resistant to ampicillin has increased in recent years from 6 to 95%. Resistance was due to R factor in 92–99%. *S. sonnei* strains isolated from patients in Sweden carried R factor in 81% [26]. The R factor carrier state is lower in bacteria isolated from healthy persons in Sweden than in other countries, probably owing to the limited use of antibiotics. Among shigellae isolated in London in the late 50's the incidence of resistant strains was less than 10%, while in 1970 more than 70% of the *S. sonnei* strains were multi-resistant. For the resistance in *S. sonnei* strains, R factor was responsible in 85.6% [28].

In Poland, 90% of 789 *S. flexneri* strains were resistant to one or more antibiotics; transfer of resistance could be proved in some selected strains [29]. In Roumania SASARMAN and SURDEANU [30] dealt with R factors isolated from *S. sonnei* and *S. flexneri*. SURDEANU *et al.* [31] examined 1200 *Shigella* strains in 1973; 45.6% were antibiotic resistant and the presence of R factors was demonstrated in more than 90% of the multi-resistant strains. SIVOLODSKY reported 96.5% R⁺ strains in *S. flexneri* [32]. In Hungary in 1972–1973, among the examined 2500 *S. sonnei* strains 61.6% proved to be resistant to antibiotics. All of the examined 75 strains carried R factor [33].

Our findings on the fi character of R factors differ from data in the literature. YOSHIKAWA *et al.* [34] found that R factors isolated from *Shigella* in Japan were fi^+ in 122 of 124 cases. We detected fi^- R factors in 87.1% by the use of male-specific phages. Though this is a world-wide used method we shall complete it with conjugation inhibition experiments in the future.

Although many fi^+ R factors confer resistance to Cm, fi^- R factors seldom do so. WATANABE *et al.* [35] stated in 1964 that all the naturally occurring fi^- R factors which they had studied lacked Cm resistance, and only in 1968 reported WATANABE *et al.* [36] an fi^- R factor (S-a) from *Shigella* which conferred Cm resistance. AOKI *et al.* [37] described R factors from the fish pathogen *Aeromonas liquefaciens* which were all fi^- and some of which carried Cm resistance determinants. HEDGES and DATTA [38] did not find fi^- factors determining Cm resistance among several hundred drug-resistant strains of enteric bacteria in England. SHAW *et al.* [39] reported an fi^- R factor which determined Cm-acetyl transferase. JONSSON [26] found among about 400 drug-resistant R^+ strains of enteric bacteria fi^- R factors responsible for Cm resistance in 16 cases. We could often observe fi^- R factors which confined Cm resistance (42 Cm, 165 Cm-Tc, 63 Sm-Cm-Tc). We suppose that ecological factors are responsible for the prevalence of various kinds of R factor in different areas.

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RESTRICTION AND MODIFICATION OF SHIGELLA FLEXNERI PHAGES BY R FACTORS

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Summary. Out of 420 R factors derived from *Shigella flexneri* strains, 50.8% restricted *Escherichia coli* and *S. flexneri* phages. Phage restriction was produced both by fi^- and fi^+ R factors. The R factors were divided into nine groups on the basis of the efficiency of plating of *S. flexneri* phages. Changes of phage types were produced by transferring R factors of different restrictive types. The changes offered some information concerning the evolution of phage types. Studies on phage modification supported the grouping of R factors determined on the basis of restriction. R factors of different restrictive types were type-specific except for types VIII and IX. Modified phages proved to be highly practical for epidemiological purposes. The use of modified phages, as an additional phage-set besides the basic phage-set, was suggested to trace the source of strains which changed their phage types as an effect of R factors.

In the phage typing of pathogenic bacteria, phage restriction by R factors is of great epidemiological importance. Though phage restriction has been studied in different bacterial species, data are not available on phage restriction in *Shigella flexneri* strains. For this reason we have studied the role of R factors in the changes of phage types of *S. flexneri* strains and characterized R factors on the basis of their restrictive effect.

WATANABE *et al.* [1] were the first to show that certain fi^- R factors, when introduced into non-lysogenic *Escherichia coli* K12, reduced the efficiency of plaque formation of phages λ , T1 and λ , T1 and T7. MOLINA [2] demonstrated genetic elements with cytoplasmic location in *Klebsiella pneumoniae* which controlled the resistance to streptomycin, tetracycline and phage T1, and could be transferred into *E. coli* K12. This author suggested, that the phenomenon was similar to that described by WATANABE *et al.*

WATANABE *et al.* [3] later observed restriction of the F^- specific phage W-31 and phage P22 in strain *Salmonella typhi-murium* LT-2. Phages λ , T1, T7 and W-31 were restricted but not modified by the fi^- R factor N-1, while phages λ , T1 and P22 were restricted and modified by the other two fi^- R factors N-3 and R15.

Phage restriction by fi^- R factors was observed independently by ANDERSON and LEWIS [4] and ANDERSON [5] in *S. typhi-murium*. When the host strain was phage type 36 of *S. typhi-murium* sensitive to all 30 of the *S. typhi-murium* typing phages, after having acquired R factor, the phage

type changed to type 6 which was sensitive only to 9 of the phages. It was shown that phage restriction was produced by the Δ transfer factor itself.

ANDERSON [5] studied the phage-restricting effect of transfer factor Δ on *S. typhi*, and *S. paratyphi-B*. A *S. typhi* strain of phage type "A" characterized by sensitivity to all Vi II phages became resistant to all Vi II phages carrying Amp- Δ , and changed to phage type 29 (lysed by 13 Vi II phages) acquiring Tc- Δ . The phage type of strain *S. paratyphi-B* changed from 1 var 2 (sensitive to 11 phages) to Beccles var. 2 (sensitive to 3 phages) after the acquisition of either Δ or T Δ .

SCHOLTENS [6] also observed phage-restricting effects of R factors in *S. typhi-murium* using a set of typing phages different from those used by ANDERSON.

GUINÉE *et al.* [7, 8] reported phage restriction by R factors in *S. panama*. In *S. panama*, phage type "A" has become less frequent than type "B" since 1960 and at the same time transmissible tetracycline-resistance has become more prevalent. The R factor with the Tc gene changes the phage type from A to B, as shown by reversion of R⁻ segregants to type A.

TSCHÄPE and RISCHE [9] demonstrated changes of phage types by fi⁻ R factors in *S. sonnei* strains. They investigated some outbreaks in which phage types changed from 3 to 9, from 5 to 70 and from 12 to a non-characteristic type. Typing with Hammarström's phages, they observed the restriction of phages VII; III, IV, VII; III, V; and V, VII. They demonstrated that phage type 9 had developed from type 3 by plasmid acquisition and emphasized the importance of changes of phage types by R factors when studying outbreaks.

Materials and methods

Strains. R factor donor strains: *S. flexneri* strains isolated from patients in Hungary in 1972-1974. Recipient strains: *E. coli* K12 Hfr, nalidixic acid resistant; *S. flexneri* "232" var. X, phage type 3, isolated from a patient.

Phages. Phage set for phage typing of *S. flexneri* strains [10]. Phage set for phage typing of *E. coli* strains [11]. Male-specific phages: *Ms*₂ [12] and *f*₂ [13] for determining fi character.

Culture media. Nutrient broth agar; eosin methylene blue agar and desoxycholate citrate agar containing streptomycin, chloramphenicol, tetracycline (30 µg/ml) and/or nalidixic acid (50 µg/ml) were used as selective media. Sensitivity to antibiotics was tested by the disk method, using "Resistest" disks (Human, Budapest) and Mueller-Hinton agar.

Crosses were carried out by the method of TSCHÄPE and RISCHE [9]. The 18 hr broth cultures of the donor and recipient strains were centrifuged, the sediments were mixed 1:1 and plated on agar plates. After incubation at 37°C for 5 hr the plates were washed with broth and the concentrated and diluted mixture was plated on selective media and incubated at 37°C for 16 hr. The colonies were subcultured three times on agar plates containing the different antibiotics.

Determination of fi character. Each R factor was classified as fi⁺ or fi⁻ according to repression of F function after transfer to *E. coli* K12 Hfr H. This was tested by examination of the Hfr R⁺ culture for visible lysis on plating with phages *Ms*₂ and *f*₂.

Phage restriction. Phage sensitivity of R⁺ and R⁻ strains was examined by routine test dilution of the adequate phage set [10, 11]. In the case of reduced lysis, the efficiency of plating (e.o.p.) was assessed by the agar layer method on *S. flexneri* strain 232 using the phages of the *S. flexneri* phage-set. The e.o.p. values were based on averages of three experiments.

Results

Changes of phage type. Examining the phage types and sensitivity to antibiotics of *S. flexneri* strains it was found that strains isolated repeatedly from the same persons and strains isolated from the same family or in the same outbreak had changed in phage type and antibiotic sensitivity. Table I shows some examples of phage types occurring in outbreaks. In these cases it could be shown that the change in phage type was due to R factors. The presence of R factors was demonstrated in the resistant *S. flexneri* strains and they were transferred to sensitive ones by conjugation. Thus naturally occurring changes in phage types were reproduced *in vitro*. It was clear from these experiments that different phage types isolated in course of the same outbreak were identical as to the source of infection.

Restriction of *E. coli* and *S. flexneri* phages. Changes of phage types developed as a result of restriction of certain phages, since there was no difference in their adsorption to R⁺ and R⁻ strains. It was therefore attempted to characterize R factors on the basis of their restrictive effect. R factors were transferred from resistant *S. flexneri* strains to a sensitive *E. coli* K12 Hfr strain and restriction of *E. coli* phages was examined. Subsequently, the R factors were transferred from the antibiotic-resistant *E. coli* to an antibiotic-sensitive *S. flexneri* strain designated 232, and restriction of *S. flexneri* phages was examined. Table II shows the restriction of *E. coli* and *S. flexneri* phages by 420 R factors isolated in the years 1972-1974. Restriction was observed in 49.4% of the fi⁻ and in 59.2% of the fi⁺ R factors. Out of the examined 420 R factors, 207 restricted neither *E. coli* nor *S. flexneri* phages. Both *S. flexneri* and *E. coli* phages were restricted by 128 R factors; *E. coli* phages were restricted by 71 and *S. flexneri* phages by 14 R factors.

In the case of *E. coli* phages the restriction of phages 23 and 24, separately and simultaneously, occurred the most frequently. These two phages are serologically related, belonging to the same serological group "D₁" [11]. Phages 5, 28, 15-23-24, 5-15-23-24, 15-16-17-18-23-24, 2-3-6-12-15-17-18 were also restricted in the given order of frequency.

Grouping of R factors on the basis of restriction of *S. flexneri* phages. Employing *S. flexneri* phages, 8 restriction-patterns were observed. E.o.p. values were measured in strain *S. flexneri* 232 carrying R factors of different restriction-pattern and compared to values assessed on strain 232 R⁻. Table III shows the relative e.o.p. values. (The original 232 R⁻ strain was lysed

Table I
Changes of phage types in strains derived from dysenteric outbreaks

Foci	Strains	Serotypes	Phage types	Phages																		
				a	F10	F3	F11	F4	D14	F9	D8	c	F5	F6	D19	F12	D2	D17	F2	b	f	d
1	R ⁻	4a	81	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
	R ⁺	4a	83	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
2	R ⁻	2a	16	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	-
	R ⁺	2a	50	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	-	-	-
3	R ⁻	3a	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
	R ⁺	3a	55	+	+	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-
4	R ⁻	3a	15	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-
	R ⁺	3a	55	+	+	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-
5	R ⁻	3a	31	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	-	-
	R ⁺	3a	66	+	+	-	-	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-
6	R ⁻	2a	16	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	-
	R ⁺	2a	59d	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	+	-	-	-

+ = lysis

- = no lysis

59d = degraded type

Table II

Distribution of R factors exerting restriction on E. coli and S. flexneri phages (1972-1974)

	No. of R factors							
	1972		1973		1974		Total	
	fi+	fi-	fi+	fi-	fi+	fi-	fi+	fi-
Phage restriction not observed	4	66	16	67	2	52	22	185
<i>E. coli</i> phages restricted	13	16	6	13	1	22	20	51
<i>S. flexneri</i> phages restricted	—	11	—	—	—	3	—	14
<i>E. coli</i> and <i>S. flexneri</i> phages restricted	2	47	8	34	2	35	12	116
Total	19	140	30	114	5	112	54	366

by 16 out of the 19 phages, but not by phages b, f and d. Strains 232 R⁺ were not lysed by these three phages either and were, therefore, omitted from Table III.)

Table III demonstrates that R factors could be divided into 9 types on the basis of e.o.p.

Type I. R factors did not restrict phages.

Type II. A single phage showed a high degree restriction. The decrease of e.o.p. by 10–10² was considered a small, the decrease of e.o.p. by 10³–10⁷, a high degree of restriction.

Type III. Two phages restricted in a small degree.

Type IV. Three phages restricted in a small degree.

Type V. Two phages restricted in high, and three in small degree.

Type VI. Seven phages restricted in high, two in small degree.

Type VII. Seven phages restricted in high, four in small degree.

Type VIII. Eight phages restricted in high, two in small degree.

Type IX. Eight phages restricted in high, three in small degree.

This grouping is consequent only with the given recipient strain; with a different recipient the restriction pattern and the degree of restriction may be different.

The presence of R factors caused modifications in plaque-morphology, too. The plaques of phage F3 are clear ones, 3 mm in diameter. Acquisition of R factor type V resulted in plaques with halo, and R factor type VII reduced the plaque size to 2 mm. The size of plaques of phages F4 and F9 were reduced by R factor type II from 7 mm to 3 and 1.5 mm, respectively.

Table III

Grouping of R factors on the basis of phage restriction. Efficiency

Types of R factors	Strains	Relative e.o.p. values					
		a	F10	F3	F11	F4	D14
	232 R ⁻	1	1	1	1	1	1
I	232 (R 901)	1	1	1	1	1	1
II	232 (R 921)	1	1	1	1	1	1
III	232 (R 496)	1	1	1	1	1	1
IV	232 (R 264)	1	10 ⁻²	1	1	10 ⁻¹	10 ⁻¹
V	232 (R 458)	1	1	7 × 10 ⁻⁵	1	1	5 × 10 ⁻¹
VI	232 (R 680)	1	1	2 × 10 ⁻⁵	10 ⁻³	3 × 10 ⁻⁴	2 × 10 ⁻⁵
VII	232 (R 557)	1	1	4 × 10 ⁻⁶	4 × 10 ⁻⁵	10 ⁻⁵	3 × 10 ⁻⁵
VIII	232 (R 296)	1	1	2 × 10 ⁻⁵	10 ⁻⁴	2 × 10 ⁻⁵	10 ⁻⁵
IX	232 (R 584)	1	1	2 × 10 ⁻⁵	10 ⁻³	2 × 10 ⁻⁵	10 ⁻⁵

Table IV shows the distribution of R factors according to restriction types.

Table IV

Distribution of S. flexneri R factors according to phage restriction types

Type of R factors	No. of R factors
I	278
II	7
III	13
IV	16
V	7
VI	25
VII	21
VIII	9
IX	44
Total	420

Table V demonstrates some examples of phage type changes due to the acquisition of R factors of different restriction type. Acquisition of R factors caused no change in the serotype of the strains.

of plating of S. flexneri phages in strain S. flexneri 232

for phages

F9	D8	c	D19	F6	F5	F12	D2	D17	F2
1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	6×10^{-6}
1	1	1	1	1	1	2×10^{-2}	2×10^{-1}	1	1
1	1	1	1	1	1	1	1	1	1
10^{-4}	1	1	1	1	1	1	3×10^{-2}	10^{-1}	1
2×10^{-5}	10^{-2}	1	1	1	1	1	4×10^{-3}	10^{-1}	10^{-6}
7×10^{-6}	3×10^{-4}	10^{-1}	1	1	1	10^{-1}	2×10^{-2}	10^{-2}	3×10^{-6}
7×10^{-6}	2×10^{-3}	1	1	1	1	5×10^{-1}	10^{-4}	6×10^{-2}	10^{-6}
7×10^{-6}	10^{-3}	10^{-2}	1	1	1	4×10^{-2}	10^{-5}	10^{-2}	10^{-6}

Phage modification. To examine the modification of phages by R factors the phages were propagated on strain 232 carrying R factors different in restrictive type and the e.o.p. values of the modified phages were assessed. Table VI presents the relative e.o.p. values for one representative each from a group of phages with identical behaviour, e.g. phage F3 from the group of modified phages F3 and F9; and phage F4 from the group containing F4, F11, D8 and D14.

Host modification was not observed when the phages were propagated in the 232 R⁻ strain.

Phage F2 and modified F2. Phage F2 was restricted by R factors of type II, IV, VII, VIII, IX. Phage F2 modified by R II had an e.o.p. = 1 on 232 (R II), but was restricted by R VI, VII, VIII, and IX. Examining F2 · R VI, the reduction in e.o.p. was less on strains 232 (R VIII) and 232 (R IX), but remained the same on strains 232 (R II) and 232 (R VII). Phage F2 · R IX was not restricted by R factors of type R IX, R VI and R VIII, and the degree of restriction was reduced by type R II and R VII.

Phage F3 and modified F3 phages. Phage F3 was restricted by R factor type R V–R IX; when modified by type R V, the restrictive effect of types R VI–R IX persisted. The e.o.p. value for phage F3 · R IX was 1 on the strains carrying R VII and R VIII, but the modified phage was restricted by R V.

Phage F4 and modified F4 phages. Modification by R IV produced no change in the reduction of e.o.p. values in the presence of R factors of type R VI–R IX, but modification by R IX protected the phage from the restrictive effect of all of the R factor types.

Table V
R factor-mediated changes of phage types in S. flexneri strains

Sero- types	Phage types	Phages																			R factor types
		a	F10	F3	F11	F4	D14	F9	D8	c	F5	F6	D19	F12	D2	D17	F2	b	f	d	
4a	89	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	+	—	—	—	II
	90	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	
3a	18	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	—	—	—	V
	50	+	+	—	+	+	+	—	+	+	+	+	+	—	+	+	—	—	—	—	
2a	69	+	—	+	+	+	+	+	+	+	+	+	+	—	+	+	—	—	—	—	VI
	74	+	—	—	—	—	—	—	+	+	+	+	+	—	+	+	—	—	—	—	
3a	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	VI
	55	+	+	—	—	—	—	—	+	+	+	+	+	+	+	+	—	—	—	—	
2a	69	+	—	+	+	+	+	+	+	+	+	+	+	—	+	+	—	—	—	—	VII
	76	+	—	—	—	—	—	—	±	—	+	+	+	—	+	+	—	—	—	—	
3a	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	IX
	58	+	+	—	—	—	—	—	+	—	+	+	+	—	±	+	—	—	—	—	
var. X	12	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	—	—	—	IX
	56	+	+	—	—	—	—	—	±	±	+	+	+	—	±	±	—	—	—	—	

+ = strong lysis

± = weak lysis

— = no lysis

Table VI

Efficiency of plating of S. flexneri phages modified by R factors

Strains	Relative e.o.p. values						
	Phages						
	F ₂				F ₃		
	Original	Modified			Original	Modified	
	F ₂	F ₂ · R II	F ₂ · R VI	F ₂ · R IX	F ₃	F ₃ · R V	F ₃ · R IX
232 (R II)	6 × 10 ⁻⁶	1	10 ⁻⁴	10 ⁻²	1	1	6 × 10 ⁻¹
232 (R III)	1	1	1	1	1	1	1
232 (R IV)	1	1	1	1	1	1	1
232 (R V)	1	1	1	1	7 × 10 ⁻⁵	1	2 × 10 ⁻⁴
232 (R VI)	10 ⁻⁶	3 × 10 ⁻⁴	1	1	2 × 10 ⁻⁵	3.5 × 10 ⁻⁶	2 × 10 ⁻¹
232 (R VII)	3 × 10 ⁻⁶	6 × 10 ⁻⁵	10 ⁻⁴	10 ⁻²	4 × 10 ⁻⁶	4 × 10 ⁻⁶	1
232 (R VIII)	10 ⁻⁶	8 × 10 ⁻⁵	10 ⁻²	1	2 × 10 ⁻⁵	4 × 10 ⁻⁶	1
232 (R IX)	10 ⁻⁶	5 × 10 ⁻⁵	2 × 10 ⁻²	1	2 × 10 ⁻⁵	4 × 10 ⁻⁶	1

Strains	Relative e.o.p. values					
	Phages					
	F ₄			F ₁₂		
	Original	Modified		Original	Modified	
	F ₄	F ₄ · R IV	F ₄ · R IX	F ₁₂	F ₁₂ · R III	F ₁₂ · R IX
232 (R II)	1	7 × 10 ⁻¹	1	1	1	1
232 (R III)	1	1	1	2 × 10 ⁻²	1	1
232 (R IV)	10 ⁻²	1	1	1	1	1
232 (R V)	1	1	1	1	1	1
232 (R VI)	3 × 10 ⁻⁴	1.2 × 10 ⁻⁵	1	1	1	1
232 (R VII)	10 ⁻⁵	1.1 × 10 ⁻⁵	1	10 ⁻¹	1	1
232 (R VIII)	2 × 10 ⁻⁵	1.2 × 10 ⁻⁵	1	5 × 10 ⁻¹	1	1
232 (R IX)	2 × 10 ⁻⁵	1.2 × 10 ⁻⁵	1	4 × 10 ⁻²	1	1

Phage F12 and modified F12 phages. Phage F12 modified by R III and R IX lysed the strain carrying whichever type of R factor in the same degree.

When strain 232 was lysogenized by the type phages, the strains did not become lysed by the modified phages.

Discussion

If plasmids are to be traced epidemiologically, they must be classified. A possibility for classification is to follow the same drug-resistance patterns [1],

but this method is not satisfactory because the same drug-resistance patterns may be determined by unrelated R plasmids.

WATANABE *et al.* [1] classified R factors according to their effect on F factor. R factors which inhibited the expression of fertility of male strains were called fi^+ , the others fi^- . These authors found that the loss of F fertility caused by fi^+ factors was accompanied by a loss of sensitivity to the male-specific phages, while fi^- factors did not affect this sensitivity. Inhibition of visible lysis by the male-specific phages thus proved to be a convenient method of monitoring the fi^+ character. We determined the fi character of R factors using male-specific phages, but this classification was not sufficient either, in view of the high frequency of fi^- R factors (87.1%).

"Superinfection immunity" and "incompatibility" between two fi^+ or two fi^- R factors offers another possibility to classify R factors. Cells with resident plasmid exclude the entry by conjugation of a second related plasmid. Related plasmids being incompatible, they cannot replicate or coexist stably in the same cell. Classification of plasmids according to compatibility has been described by DATTA and HEDGES [15-20]. The plasmids which determine F pili constitute five compatibility groups (FI-FV). Plasmids which determine I pili belong to superinfection immunity group I and are divided into five compatibility groups; fi^- R plasmids belong into further groups: N, P, W, and O, C, A, M, T. Group H contains fi^+ plasmids which do not determine F pili.

CHABBERT *et al.* [21, 22] studied the incompatibility phenomenon and confirmed the existence of groups I and N (DATTA and HEDGES) and described five other groups designated 5, 6, 7, 8, 9. They have suggested the use of other symbols: plasmid N3 which belongs to group N according to DATTA and HEDGES, appears as R_{N_3} com 2 str tetA sul.

We have decided to study in the future the incompatibility phenomena among the naturally occurring R factors, because these experiments may provide a reliable method of R factor classification and allow conclusions as to the true phylogenetic relationships. This way of grouping R factors is, however, of no help in determining phage type changes. For this purpose it seemed advisable to examine the restriction by R factors. This idea was supported by the report of ANDERSON [23] who suggested to determine bacteriophage restriction in salmonella hosts for subdivision of R factors and transfer factors.

Subdivision of R factors based on phage restriction proved to be useful. The changes of phage types *in vitro* due to different restrictive R factor types provided some evidence on the evolution of phage types. The hypothesis that phage types with limited phage sensitivity developed from types with wide lytic spectra after R plasmid acquisition was supported by the experiments.

For example phage type 55, which occurs in our material since 1971 as a frequent type among the strains of serotype 3a, may have developed from phage type 3. Phage types 50, 54, 64, 66 and 68 probably developed from "antibiotic-sensitive" types 5, 18, 19 and 31. Among strains of serotype 2a, from the "antibiotic-sensitive types" 14, 16, 69 developed the "antibiotic-resistant types" 73, 74 and 76. Phage type 90 originated from types 81 and 89, which were frequent among serotypes 4a. Examining the fertility inhibition properties by testing the Hfr R⁺ culture for visible lysis on plating with F phage (Ms₂), both fi⁺ and fi⁻ R factors restricted phages. There is a contradiction in the literature on phage restriction by fi⁺ R factors. According to the early reports, phages were restricted only by fi⁻ R factors [1, 9] but restriction by fi⁺ R factors has also been reported. SICCARDI [24] demonstrated restriction of phages W31 and BF23 in *E. coli* K12, and this was not confined to the fi⁻ R factors alone. BANNISTER and GLOVER [25] surveyed 151 R factors both fi⁺ and fi⁻ in the *E. coli* K12 strain for restriction of 8 phages. They observed restriction by both fi types of R factors. R factors were divided into ten groups on the basis of the e.o.p. value. HEDGES and DATTA [19] reported a fi⁺ R factor, designated R 124, which conferred tetracycline resistance and restricted and modified DNA phages, λ phage included. The specificity of restriction was designated hsp I. BANNISTER then observed [26] that fi⁺ and fi⁻ R factors were present in the same strain and the fi⁺ R factor could mask the presence of the fi⁻ R factor. The restrictive effect of fi⁺ R factors was evident in our experiments. Masked fi⁻ R factors were not present as phage restriction was examined after two crosses, and in these cases the presence of two kinds of R factor was not observed, and all the resistance determinants were transferred to the final host, too.

The modification experiments supported the grouping of R factors based on phage restriction; only types R VIII and R IX seemed to belong to one type.

The modification by R factor type II protected the restriction only by R factor type II. Modifications by R IV, R V and R VI were also type specific. Modification by type R IX protected the restriction by R factors of types R IX and R VIII, and to a smaller extent by type R VII, too.

The experiments on modification resulted in practical advantages in phage typing. When different phage types are isolated from the same outbreak, to find the source of infection some additional method must be used as R factor transfer needs much time and so does not meet the main requirement of quick typing. By the help of the additional modified phage set one can deduce the original phage type of the R⁻ strain if the strains carrying different types of R factor are not lysed by the type phages but are lysed by some of the modified phages.

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ENDOTOXIN-INDUCED NON-SPECIFIC RESISTANCE IN RATS EXAMINED BY TRYPANOSOMA EQUIPERDUM CHALLENGE

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Summary. In rats weighing 100 g, intravenously administered *Trypanosoma equiperdum* organisms started logarithmic growth immediately, whereas in rats weighing 150–300 g growth started after a lag phase of 0.7–3.1 hr. The lag phase lasted for 15–17 hr when the rats were pretreated serially with endotoxin and this time course was not modified by changes either in the host's body weight or in the germ count of the inocula. Maximum resistance to infection was achieved with gradual doses of the endotoxin given at 48 hr intervals on 6 occasions. The total dose of endotoxin was fourfold of its LD₅₀. Additional doses failed further to increase the resistance. Serial passages of the strain in rats pretreated with endotoxin disclosed that the trypanosomes growing after the lag phase had developed resistance to the factor responsible for the lag phase. Thus the method does not allow a quantitative estimation of resistance induced by serial endotoxin treatment, yet it represents a rapid and simple procedure in every case in which qualitative assessment is sufficient.

Experimental animals pretreated with endotoxin are known to show increased non-specific resistance to bacterial [1–4], viral [5, 6], fungal [7–9] or to parasitic infections [10–13]. It was shown earlier that trypanosomes administered intravenously to rats 48 hr after either a single or serial endotoxin treatment started to grow only after a lag phase of varying duration [14]. The doubling time of the trypanosomes was, according to MÜHLFORDT's data, 5.5 hr, in both endotoxin-treated and control rats [15].

The finding that in rats treated with a single endotoxin injection, the lag phase could be prolonged by elevation of the dose suggested that the length of the lag phase might be conclusive also of quantitative relations [14]. Nevertheless, the maximum duration of the lag phase inducible with a single injection was 7 hr, even with a dose as high as 450 µg/100 g.

The primary aim of the present studies was to clarify the extent to which the total dose of serially administered endotoxin, the number of treatments, body weight of the rats and the germ count of the inoculum might influence the length of the lag phase.

Materials and methods

Experimental animals. Wistar male rats, weighing 100–300 g were used throughout.

Endotoxin. *Serratia marcescens* endotoxin was prepared by the method of BOIVIN and MESROBEANU; 450 µg/100 g of the preparation corresponded to the LD₅₀.

For treatment, the endotoxin was given in rising doses (50, 100, 200, 400, 800 and 1600 µg/100 g) at 48 hr intervals on 6 occasions. Challenge with *Trypanosoma equiperdum* was performed 48 hr after the last endotoxin dose.

Challenge. A *Trypanosoma equiperdum* strain maintained in this laboratory in mouse passages since 1942 was used. The rats were challenged intravenously with doses adjusted so as to assure a given initial germ count in the circulating blood. The technique of growth curve analysis was described in a previous paper [14].

Results

In the first series of experiments, the relationship between the total dose of endotoxin used for serial treatment and the length of the lag phase were examined.

Rats weighing 100–200 g were challenged intravenously with trypanosomes after the first, second, third, etc., endotoxin treatment, with inocula adjusted to assure a blood level of 2×10^7 organisms per ml.

The duration of the lag phase established from the growth curves is shown in Table I. The length of the lag phase tended to increase steadily

Table I

Relationship between number of endotoxin treatments and length of lag phases of trypanosomal growth curves

Applied doses of endotoxin ($\mu\text{g}/100 \text{ g}$)	No. of animals	Length of lag phase, hr	SEM
50	8	2.1*	0.24
50, 100	6	7.3*	0.86
50, 100, 200	6	10.6*	0.86
50, 100, 200, 400	7	14.2*	1.15
50, 100, 200, 400, 800	8	16.5*	1.05
50, 100, 200, 400, 800, 1600	11	16.2	1.42

Note: Statistical analyses were performed by Student's method

* The difference between any of the 5 groups is significant ($p < 0.01$)

after the first four treatments, but from the fifth treatment on, no further prolongation could be achieved by endotoxin.

In the next series, the effect of body weight of the rats on the length of the lag phase was examined. Rats weighing 100–300 g, untreated or treated 6 times with endotoxin as described above, were challenged intravenously with trypanosomes, using an initial germ count of 2×10^7 per ml blood.

Results are summarized in Table II. Appearance of a lag phase was noted with the untreated control rats weighing more than 150 g. With the body weight group of 300 g, lag phases of 2–3 hr duration were measured regularly. With the endotoxin treated rats, higher body weights had apparently no influence on the length of the lag phase.

Table II

Relationship between body weight of hosts treated and not treated with endotoxin and length of lag phases of trypanosomal growth curves

Untreated control				Treated 6 times with endotoxin			
Body weight of animals, g	No. of animals	Length of lag phase, hr*	S.E.	Body weight of animals, g	No. of animals	Length of lag phase, hr**	SEM
100	10	0	—	100	48	16.2	1.40
150	18	0.7	0.08	150	24	15.8	1.05
200	9	1.1	0.16	200	8	17.4	1.16
250	6	1.9	0.14	250	6	15.6	1.24
300	6	3.1	0.30	300	6	16.4	1.11

* The difference between any of the 4 groups is significant ($p < 0.01$)

** There is no significant difference between any of the 4 groups ($p < 0.1$)

It was therefore concluded that with the particular trypanosomal and rat strains used in the present study, lag phases of 15–18 hr duration represent the upper limit of the organism's capacity for resistance, regardless of the nature of the mechanism by which the resistance develops.

In principle, the upper limit of resistance capacity may be determined either by an exhaustion of the organism's resources or by the development of resistance in the applied trypanosomal strain. If the first alternative were true, a notable increase of the infective dose should accelerate the decline of resistance.

Rats weighing 100 g and pretreated 6 times with endotoxin were challenged intravenously with inocula adjusted to make initial trypanosomal counts of 2×10^7 , 4×10^7 , 8×10^7 or 16×10^7 per ml blood. The lengths of the lag phases read from the growth curves are shown in Table III.

Table III

Relationship between trypanosomal counts of infectious doses and length of lag phases of trypanosomal growth curves

Initial germ count/ml	No. of animals	Length of lag phase, hr*	SEM
10^7	6	15.8	1.18
2×10^7	21	16.2	1.24
4×10^7	14	17.4	1.06
8×10^7	8	16.1	1.32
16×10^7	8	17.1	1.16

* There is no significant difference between any of the 5 groups ($p < 0.1$)

Apparently, an increase in the inoculum's germ count failed to abbreviate the lag phase. This supported the assumption that in rats pretreated with endotoxin the limiting factor of the lag phase is not so much the exhaustion of the host organism as the development of resistance by the trypanosomes.

Part of the trypanosomes applied by us may also have been resistant against the lag phase-inducing effect. To prove this hypothesis, the trypanosomal strain was passaged in rats serially pretreated with endotoxin; intravenous transfers were always made from the post-lag logarithmic phase. The length of the lag phase was determined after each passage. Rat weighing 100 g were pretreated with endotoxin 6 times. Growth curves were derived 48 hr after the last endotoxin treatment. The transferred inocula were adjusted so as to assure an initial germ count of 2×10^7 trypanosomes per 1 ml blood.

Table IV

Decrease of lag phase of trypanosomal growth curves during intravenous transfer in rats pretreated with endotoxin

Passage number	No. of animals	Length of lag phase, hr	SEM
Original strain	18	16.3*	1.18
Passage I	8	5.6*	0.24
Passage II	6	2.4*	0.22
Passage III	5	1.8*	0.16
Passage IV	6	1.5	0.21
Passage V	6	1.6	0.06
Passage VI	8	1.5	0.14

* The difference between any of the 4 groups is significant ($p < 0.01$)

As can be seen from Table IV, after the first passage in endotoxin-treated rats, the length of the lag phase was reduced to one third of its original duration and after the third passage it amounted to as little as 1.8 hr, which period could not be reduced further by subsequent passages.

Obviously, in the course of transfer in endotoxin treated rats the trypanosomes had developed resistance to the factor responsible for the occurrence of the lag phase.

Discussion

Since in a previous study the lag phase measured after a single intravenous injection of endotoxin could be prolonged by increasing the dose of endotoxin, the possibility was considered that endotoxin-induced resistance

to trypanosomal challenge might be estimated quantitatively on the basis of the length of the lag phase. It remained to be clarified whether this apparent quantitative relationship was valid with serial instead of a single endotoxin treatment. For this purpose, the effect on the length of the lag phase of the hosts' body weight, the germ count of the inoculum and the total dose of endotoxin was examined to allow a reliable estimation of the eventual relationship.

Minor changes of the animals's body weight and of the inoculum's germ count failed to influence the length of the lag phase, while the total dose of endotoxin and the number of treatments influenced it considerably, though neither of them could prolong it beyond a certain limit.

Lag phases not longer than 10–11 hr were measured even after the 8th treatment in rats pretreated with 100 μ g/100 g endotoxin every 48 hr. The extent of the resistance seemed to be determined by the administered endotoxin doses rather than by the number of treatments (unpublished data).

The fact that the maximum resistance inducible with endotoxin could not be reduced by increasing the germ count of the challenge dose, nor increased by using rats of a higher body weight, suggested that beyond a given weight range of the host and a given dose range of trypanosomes the length of the endotoxin-related lag phase of trypanosomal growth was no longer conclusive of the extent of the host's resistance. This conclusion was supported by the results of serial transfers of trypanosomes in rats pretreated serially with endotoxin.

The factor responsible for the lag phase has not been identified, but of whatever nature it might be, it is certain that the trypanosomes growing after the lag phase had developed resistance to it. Once this type of resistance had been established, the applied *Trypanosoma equiperdum* strain was no longer suitable for the quantitative estimation of the host's endotoxin-induced resistance. Similar limitations by the test organism of a phenomenon's quantitative evaluation have been observed also by others [16]. Nevertheless, the method itself has been reliable in those cases in which the mechanism of the endotoxin-related increase of resistance had to be rapidly estimated on a qualitative basis, using a small number of animals.

In accordance with BERGER [17] we have found that serial endotoxin treatment induces in tolerant animals a higher resistance than a single endotoxin treatment. This finding is in contradiction with the results reported by MC GREGOR *et al.* [13].

The dose dependence of the lag phase after a single intravenous injection of endotoxin is explained by the development of a much lower, yet dose-dependent resistance, the decline of which is actually indicated by the termina-

tion of the lag phase. In contrast, in rats serially treated with endotoxin the lag phase comes to an end when the resistant trypanosomes become predominant over the non-resistant population.

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STUDIES ON MEGACINOGENY IN *BACILLUS CEREUS*

I. MULTIPLICATION OF PHAGE *wx* CAUSING LYSOGENIC CONVERSION TO MEGACIN A (PHOSPHOLIPASE A) PRODUCTION

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Summary. Phage *wx* capable of reconverting *Bacillus cereus* strain W derivatives, cured to lose megacin A (phospholipase A) production into megacin A-producing cultures, exhibits unusual kinetics of multiplication; its clear mutant, phage *wxc*, behaves similarly. The phages are not adsorbed by stationary phase indicator bacteria. As sonicated bacteria fail to inactivate the phages, the absence of adsorption cannot be attributed to an under-surface localization of the receptors. Multiplying bacteria exert a slow and slight degree of phage adsorption. Cells inhibited by chloramphenicol produce no receptors. It has been assumed that the receptor, produced from a precursor involved in bacterial cell synthesis, either absorbs the phage in the nascent state or is incorporated in the cell and loses its phage-adsorbing capacity.

Two decades ago we described a bacteriocin produced by about 5% of *Bacillus megaterium* strains [1, 2]. As *B. megaterium* produces several bacteriocins, this factor was, upon the recommendation of HOLLAND and ROBERTS [3] named megacin A. The characteristics of megacin A were reviewed in 1965 [4]. After ultraviolet irradiation, lysates of *B. megaterium* strain 216 contained high amounts of megacin A. This strain has been studied in detail [4] and is regarded as the prototype of megacin A producing cultures. The finding of OZAKI *et al.* [5], that megacin A of strain 216 is identical with phospholipase A, has been an important step in elucidation of the problem. We have assumed that information for megacin A production is associated with a prophage [6]. In subsequent studies it has been shown that lysates of megacin A producing strains induced with mitomycin C (MC) contain one or more different kinds of phage [7]. We have, however, been unable to elucidate the characteristics of the phages or prophages involved in megacin A production (unpublished data).

Later it has been shown that certain strains of *Bacillus cereus* also produce megacin A [8]. One of these strains, *B. cereus* W produces after MC induction a highly specific temperate phage termed *wx* [9]. If the strain was cultured in the presence of ethidium bromide, its derivatives lost these cured derivatives, named *cin⁻* bacteria [9], were lysogenized with phage *wx*, they regained their megacin A-producing ability. Thus, bacteriocin production may be regarded as lysogenic conversion. Phage *wx* and its clear mutant, *wxc*, ex-

hibited an unusual behaviour: whereas phages are, as a rule, adsorbed within minutes, even a partial adsorption of *wx* and *wxc* took several hours. In the presence of excess phage, infection of the whole bacterial population took 10–12 hr.

Converting phage *wx* and its clear mutant were highly specific: they failed to act on 100 strains belonging to different species of the genus *Bacillus*; they produced plaques only on cured derivatives of *B. cereus* W. It should be noted that *cin*⁻ derivatives produced no megacin A after treatment with MC [9].

Phages *wx* and *wxc* have been characterized in previous studies [9, 10], but these failed to clarify several problems such as the adsorption and multiplication of the phages. Phage *wx* was present only if *B. cereus* W had been induced with MC in liquid medium; under this condition the lysates contained approximately 10⁶ p.f.u. per ml. In liquid medium the phages were not adsorbed on the bacteria and *wxc* began to multiply only after 8–12 hr, whereas by the soft agar layer technique it produced distinct plaques 2–8 mm in diameter and, after extraction of the soft agar, it showed a titre of at least 10¹⁰ p.f.u. per ml. In the present study answers were sought to these problems.

Materials and methods

Bacterial strains. *B. cereus* strain W and its *cin*⁻ derivatives including a streptomycin resistant one (*cin*⁻ *str*), were used. Titration of megacin A was performed with strain *B. megaterium* MUT-C [8].

Phages. Preparation of phages *wx* and *wxc* was described earlier [9]. If necessary, the phage material was concentrated by the method of YAMAMOTO *et al.* [11] and filtered through membrane filter (0.45 µm).

Determination of p.f.u. YP medium plates prepared with 1.5% agar were overlaid with the following mixture kept at 47°C: phage dilution, 0.1 ml; *cin*⁻ shaken culture (OD₆₀₀ = 0.2 in YP medium), 1 ml; above YP medium, 0.9 ml. The plates were incubated at 37°C overnight.

Culture media. YP and BPY_{e1} media were described in reference [10].

Culturing of bacteria. Ten ml amounts of culture medium were distributed into 100 ml Erlenmeyer flasks and, unless otherwise indicated, shaken in a water bath at 37°C.

Determination of phage adsorption. When shaken cultures of *cin*⁻ bacteria reached an OD₆₀₀ = 0.25 (4 × 10⁷/ml c.f.u. or 2 × 10⁸/ml bacteria estimated on the basis of the average chain former), the suspension was infected with 0.1–1 m.o.i. phage. At intervals the p.f.u. of free phage in the supernatant was determined.

Lysogenic conversion was performed as described earlier [10].

Antiphage serum was prepared as in our previous paper [9]; K value = 45.

Results

Behaviour of *B. cereus* W and its *cin*⁻ derivatives under different cultural conditions. Phage *wxc* gave distinct plaques on soft agar inoculated with *cin*⁻ bacteria. After 16 hr incubation the culture caused the medium to shift from pH 7.2 to 8.5–9.0.

In liquid medium strain W and its *cin*⁻ derivative behaved in a different manner (Table I). The results of these preliminary experiments are detailed in order to point out the difference in intermediary metabolism depending on different pH values associated with the cultural conditions.

Table I

The multiplication of cin⁻ bacteria in liquid and agar medium

Medium	Way of incubation	Final pH***
Liquid YP*	static	7.0
Liquid YP*	shaking	7.0
YP agar (1.5%)**	surface inoculation	8.5
YP agar (0.75%)	in soft layer	8.5
BPYe ₁ agar (1.5%)**	surface inoculation	9.0
BPYe ₁ agar (0.75%)	in soft layer	9.0

* 10 ml of liquid medium in a 100 ml Erlenmeyer flask

** Agar plate in a Petri dish

*** The final value was determined with indicator paper after incubation overnight

Adsorption of phages. To liquid *cin*⁻ cultures phage *wxc* (m.o.i. = 1) was added. Adsorption was not detected at different electrolyte concentrations and at pH values of 5.5 to 8.0 in medium BPYe₁.

Accordingly, *B. cereus cin*⁻ derivatives appeared to be poor in receptor content. To prove this assumption, dense bacterial suspension cultured on agar surface at 37°C or 20°C (pH 9; OD = 1.2 at 1 : 10 dilution) was used in adsorption experiments. Measurable phage adsorption, however, was not detected even after 3 hr.

In view of the very slight degree of adsorption, it has been assumed that most of the receptors are not located on the cell surface. It has been shown that a polypeptide layer (T layer) on the cell surface [12, 13] is characteristic of part of the species of the genus *Bacillus* including *B. cereus* [14]. The T layer is bound to the cell surface by hydrogen bonds and can be removed with 6 M urea. Urea-treated suspensions, however, failed to adsorb phage *wxc*. Heating at 60–80°C for 30 min and sonication (supposed to release the deeply located receptors) were similarly ineffective.

In certain experiments 100 m.o.i. of phage was used and adsorption was determined indirectly; the phage-bacterium mixture was left to stand for 1 hr, then antiphage serum was added and the number of infective centres was determined on *cin*⁻ and *cin*⁻ *str* bacteria. The percentage of non-neutralized

phages was only 1% and in 6 different experiments the number of infected cells ranged between 10 and 50%.

Multiplication of phages in liquid media. As phage adsorption was not detectable even after several hours, the one step growth technique could not be used. Only *cin*⁻ bacteria growing in liquid media were suitable for examining phage multiplication. Phage *wx* (0.1–1 m.o.i.) failed to show multiplication in liquid *cin*⁻ cultures after 6–8 hr. After 24 hr the supernatant contained no phages; a few per cent of the population was lysogenic; this means that by that time all *wx* phages had, as prophages, been incorporated into the cells.

Multiplication of phage *wxc* in liquid BPY_{e1} medium was influenced by different factors (Table II). After 6 hr the phage showed a definite multiplication and by the 16th hr not only the phage titre increased but other changes had also occurred. It is interesting that the static culture showed no change in pH, whereas the aerated culture became considerably alkaline.

Kinetics of conversion. As adsorption of phages *wx* took place slowly and was slight in degree, its measurement had a considerable source of error. A study of the kinetics of adsorption was expected to give more accurate results. In previous experiments [10] the conversion of *cin*⁻ population was shown to occur gradually. From Table II in the paper cited [10], it was evident that in the presence of 0.01 M KCN at 37°C conversion took place considerably slower than in the absence of KCN. By the end of the experiment (16 hr), however, all cells had produced megacin A. As KCN fails to exert a long-term inhibition, the experiment was repeated at 22°C, with and without the addition of chloramphenicol. The conversion of *cin*⁻ population under the effect of phage *wx*

Table II

Propagation of phage wxc by cin⁻ bacteria in BPY_{e1} liquid medium under static and aerated conditions at 37°C

Condition	Different parameters	Time of incubation (hr)				
		0	3	6	8	16
Static	OD*	0.1	0.35	0.62	0.75	0.35
	pH	7.2	7.2	7.2	7.2	7.2
	p.f.u./ml	1.2×10^7	1.2×10^7	4×10^8	4×10^{10}	8×10^{10}
Shaken	OD*	0.1	0.62	1.50	1.50	0.75
	pH	7.2	7.2	8.0	9.0	9.0
	p.f.u./ml	2×10^7	3×10^7	6×10^9	1.2×10^{11}	3×10^{11}

* OD of bacterial culture determined in Bausch–Lomb 20 photometer

Table III

*Conversion rate of cin⁻ bacteria in the presence of phage wx (m.o.i.: 5)
and number of colony formers in the system*

Chloramphenicol concentration	Conversion and colony formers	Time in hours (22°C)			
		0	3	5	24
0	Conversion (%)*	2	8	32	100
	No. of colonies/ml**	3.5×10^7	5×10^7	6.5×10^7	2.3×10^8
250 µg/ml	Conversion (%)	0	0	0.5	4
	No. of colonies/ml	4×10^7	4.1×10^7	3.9×10^7	4.2×10^7

* Samples were taken at intervals, mixed to antiphage serum and explanted with indicator bacteria (Mut-C) in soft agar (see reference [10])

** Aliquots were streaked on agar plates

is shown in Table III. The rate of conversion means the percentage of *cin⁻* bacteria producing megacin A at the intervals indicated. Results are evaluated in the Discussion.

Discussion

Phage *wx* exhibits peculiar kinetics of multiplication. The slow course of its adsorption is striking. Infection of all bacteria did not occur in liquid medium even when the cell : phage ratio was 1 : 100. A valid determination of the degree of adsorption of phage *wx* was performed on the basis of the conversion of *cin⁻* cells.

The clear mutant (*wxc*) showed a slow gradual adsorption. The titre of the mutant in 16–25 hr cultures was higher by 3–4 exponents as compared to the starting value, thus the multiplication of *wxc* was confirmed in liquid culture, too. The peculiar characteristics of *B. cereus* cultures have been interpreted on the basis of changes in pH during cultivation. Under different cultural conditions the cells exhibited a change in intermediary metabolism. As to the nature of the receptors for *wx* phages, the results summarized in Table III are of interest. The presence of the receptor on the cell surface is negligible; in resting cells the adsorption is practically nil. Experiments on the kinetics of conversion give an account of the production of the receptor. If bacterial multiplication was inhibited with chloramphenicol, the number of megacin A producing cells was very low (4%) even after 24 hr. In connection with this finding, the number of colony forming units showed no alteration in 24 hr. Moderately growing bacteria in the presence of phage *wx* (in static cultures at 22°C) gradually changed into megacin A producing cells. The explanation of this finding must be that the receptor for phage *wx* is produced

during multiplication and is present only in this stage of growth. The receptor precursor, when it develops into a receptor, adsorbs the phage in the nascent state. If adsorption fails to occur, the receptor is incorporated into the cell and loses its phage-adsorbing capacity. The nature of the precursor is unknown; it may belong to the cytoplasmic membrane of the dividing cell and may be one of the precursors of the cell wall constituents.

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STUDIES ON MEGACINOGENY IN *BACILLUS CEREUS*

II. *BACILLUS CEREUS* ISOLATES CHARACTERIZED BY PROPHAGE-CONTROLLED PRODUCTION OF MEGACIN A (PHOSPHOLIPASE A)

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Summary. Five out of a number of *Bacillus cereus* strains isolated from soil produced high titre specific bacteriocin (megacin A) in mitomycin C-induced cultures. In the course of cultivation with ethidium bromide, the strains gave off segregants not producing bacteriocin (*cin*⁻). The lysate of two wild strains formed plaques on the corresponding *cin*⁻ bacteria. The two phages (*w*x23 and *w*x26) were identical in antigenic structure with phage *w*x present in the lysate of *B. cereus* strain W, and converted *cin*⁻ derivatives into cultures producing megacin A (phospholipase A). The phages produced plaques at 26°C but not at 37°C. In the lysates of the remaining three strains phages were not detected with biological and morphological methods; these cultures have been assumed to carry defective prophage genome. As the corresponding prophages are responsible for the determination of inducible phospholipase A production, phages named *w*x seem to form a separate group of *B. cereus* phages.

Strain *Bacillus cereus* W, isolated by McCLOY [1], produces after UV or mitomycin C (MC) induction a bacteriocin identical in antibacterial spectrum with megacin A (phospholipase A) [2]. In the lysate of the strain two phages (*w* and *w*x) have been detected with appropriate indicator cultures [3]. Phage *w*x carries the information for phospholipase A production, thus it corresponds to a converting phage [4].

Among several dozens of *B. cereus* strains maintained in our collection, beside strain W, only one, V8, contained in its lysate a substantial amount of megacin A together with phage *w* [2]. As V8 is an old laboratory strain, contamination with *B. cereus* W strain could not be excluded. In the present work it has been studied (i) whether megacin A-producing cultures can be isolated from soil; (ii) whether these cultures contain a prophage responsible for megacin A production.

Materials and methods

Phages and bacterial strains. Phages isolated from *B. cereus* strains were designated with the registration number of the corresponding strain. As the phages were very similar in properties to phage *w*x of *B. cereus* W, the latter was regarded as the prototype of phages converting to phospholipase A production. Accordingly, the converting phages isolated from *B. cereus* strains 23 and 26 were named *w*x23 and *w*x26, respectively.

Plaque production by the phages was examined on a number of strains belonging to different species of the genus *Bacillus*: 40 *B. cereus*, 13 *B. thuringiensis*, 5 *B. licheniformis*, 4 *B. subtilis* (Marburg), 5 *B. atterimus* and 5 *B. niger* [3]. For the detection of anthrax-specific phages, *B. anthracis* strains Sterne and Davis were used as indicators.

Culture media and phage buffer. Medium YP was prepared as described earlier [5]. Phage titration was carried out on BPYe₁ medium [3]. Megacin A (phospholipase A) was isolated from cultures grown in BCM medium prepared with 0.1% yeast extract and supplemented with thiamine (1 µg/ml) and glucose (0.2%) [4]. The concentrated phage material was suspended in the buffer of RABUSSAY *et al.* [6].

Preparation of phage material and titration of phages. MC-induced lysates of *B. cereus* wild strains were collected from plaques formed on the homologous *cin*⁻ cultures by scraping off the soft agar layer into 5 ml medium. After centrifugation the supernatant was filtered and mixed to a soft agar suspension of the corresponding *cin*⁻ bacteria and poured over solid agar plate as described in the preceding paper [7]. Incubation was done at 26°C for 24 hr, when necessary, the phage material was concentrated as described by YAMAMOTO *et al.* [8]. In this manner 10⁸ p.f.u./ml phage material was produced.

Phage sensitivity of strains. The phages were dropped at 10⁻¹, 10⁻² and 10⁻³ dilution onto soft agar layer containing the bacteria.

Cured isolates. The *cin*⁻ derivatives of bacteriocin-producing wild strains were obtained by culturing the latter in the presence of ethidium bromide until sporulation had occurred. Derivatives not producing bacteriocin were isolated from the spore suspension as described in reference [3].

Assay of megacin A production. The cultures were shaken in 100 ml yeast extract-BCM medium in 1 litre Erlenmeyer flasks at 37°C on Gyrotory Shaker (New Brunswick Scientific Co.) at 350 r.p.m. until OD₆₂₀ = 0.25 had been reached. Then 0.5 µg MC was added per ml medium and the culture was incubated until lysis had occurred. After centrifugation at 10 000 g and 4°C for 30 min in the MSE high speed centrifuge, to the clear supernatant ammonium sulphate was added to 0.7 saturation under continuous correction of the pH to 7.0. After the ammonium sulphate had completely dissolved, the liquid was refrigerated for 1 hr. The precipitate was centrifuged at 15 000 g and the deposit was dissolved in 2 ml of 0.75 M NaCl and the insoluble fraction was removed by centrifugation. Megacin A titre of the concentrates was determined as described elsewhere [2] on indicator strain *B. megaterium* MUT-C.

Phospholipase activity in the concentrated lysates was measured with the hydroxamate method [4] modified as follows. As substrate, L-α-lecithin (synthetic, dipalmitoyl, Fluka) was used at 1 µg/ml concentration in 1 ml assay system. On the basis of preliminary experiments, 3–4 units of enzyme were added to the system in 0.1 ml volume; to two other assay systems a half and a quarter of this amount were added. Hydrolysed ester bonds were expressed in µEq calculated with the formula used in previous experiments [4]. One unit of the enzyme corresponded to an amount breaking down one µEq ester bond at 37°C in 30 min. Specific enzyme activity calculated for 1 mg protein was measured by the method of LOWRY *et al.* [9].

Enzyme activity of the concentrates was identified by thin layer chromatography. Phosphatidyl ethanolamine (synthetic dipalmitoyl, A grade, Calbiochem) was used as substrate. Identification of phospholipase A activity was based on the method of GALLAI-HATCHARD and THOMPSON [10] modified as follows. The substrate was emulsified at 1 mg/ml in 0.05 M Tris buffer (pH 8.0) supplemented with sodium deoxycholate (250 µg/ml) and CaCl₂ (10 mM), in an MSE Ultrasonic Power Unit. All concentrates were tested in three assay systems containing the enzyme in gradually decreasing amounts. To 0.5 ml emulsion 2–3 units of enzyme concentrate were added in 0.1 ml volume; the second and third assay systems contained half and quarter of this amount, respectively. Incubation at 37°C lasted for 30 min, then chloroform extraction and other procedures were performed as described by GALLAI-HATCHARD and THOMPSON [10]. Chromatography was done in Silica gel G (Merck) thin layer (0.25 mm).

Lysogenic conversion. The lysate prepared from the parent strain was dropped undiluted and diluted to 10⁻¹ and 10⁻² onto soft agar layer inoculated with the homologous *cin*⁻ bacteria. The culture was incubated at 26°C for 24–48 hr, then the plaques were suspended in a few ml of saline and heated at 75°C for 10 min. Gradual dilutions of the heated spores were added in 0.1 ml volume to a mixture containing 1 ml *B. megaterium* MUT-C culture and 1 ml agar. After incubation at 37°C the ratio of colonies with and without clear zone was calculated. In liquid medium the time course of conversion was determined as described in [4], except that the system was incubated at 22–24°C. All experiments were performed in parallel in systems containing chloramphenicol (250 µg/ml).

Electron microscopic examination. Phages *wx23* and *wx26*, propagated on soft agar and extracted, were centrifuged and filtered. The extract was concentrated as described by YAMAMOTO *et al.* [8] then purified with cyclic ultracentrifugation. *B. cereus* strains 22, 27

and 28, which failed to give plaques on the homologous *cin*⁻ strains, were induced with MC, filtered and concentrated. After ultracentrifugation the material was suspended in one hundredth of the original volume and mounted on carbon-impregnated Formvar-film placed on LKB grids. The preparations were stained with 2% uranyl acetate and examined in a JEOL 100 B electron microscope at 80 kV.

Results

Isolation of B. cereus strains and screening for megacin A production.

The strains isolated from soil specimens were selected on the basis of colonial morphology. After needle-point inoculation onto agar plates the cultures were grown at room temperature for 12–16 hr then replicated by the velveteen stamp method and irradiated with UV light for 15 s. After incubation at 37°C, colonies with clear zone were reisolated and examined for morphological and biochemical characteristics as described by SMITH *et al.* [11] and for minimum nutrient requirement [12]. Dozens of isolates showing the characteristics of *B. cereus* were examined for bacteriocin production in the presence of MC. The majority produced bacteriocin in slight amounts; 5 strains producing more than 400 U/ml were studied further. Strain *B. cereus* W was used as control.

Segregants producing no bacteriocin. Derivatives were obtained from *B. cereus* isolates 22, 23, 26, 27 and 28 by culturing them in the presence of ethidium bromide [3]. These “cured” derivatives were designated as *cin*⁻ cultures.

Phages detectable in the lysates of B. cereus strains. The lysate of prototype strain *B. cereus* W in addition to converting phage *wx*, contains phage β which acts on *B. anthracis* strains [3]. Only two of the 5 *B. cereus* strains selected on the basis of bacteriocin production were shown to contain in their lysates converting phages (*wx*23 and *wx*26). The two phages were characterized by peculiar thermosensitivity. At 37°C they failed to produce plaques on the homologous *cin*⁻ bacteria. The optimum temperature for plaque production was 26°C. It was striking that plaque production was absent even at 26°C if the phages had been incubated at 37°C in soft agar layer together with the indicator culture. Accordingly, it may be assumed that phages *wx*23 and *wx*26 are thermosensitive, although their vegetative forms tolerated higher temperatures well: at 45°C for 60 min the phage lysates showed no decrease in titre.

In the lysates of three *B. cereus* strains slight amounts of certain phages were also detected; these failed to act on *B. anthracis* Davis, but produced plaques on *B. anthracis* Sterne (CN 18-74). These phages will be described in a subsequent paper.

Phages *wx*23 and *wx*26 exerted no action on various *Bacillus* strains either at 37°C or at 26°C.

On BPYe₁ medium inoculated with the homologous *cin*⁻ strains, phages *wx23* and *wx26* produced turbid plaques about 1 mm in diameter with pin-point-sized clear centre. Both phages produced a few, seemingly clear plaques which, however, yielded no clear mutants on subcultivation.

Phages *wx23* and *wx26* were closely similar in antigenic structure as phages *wx* and *wxc* of *B. cereus* W. The K value for the immune serum prepared with phage *wxc* was 45 against the homologous phage; the corresponding values for phages *wx23* and *wx26* were 43 and 41, respectively. Subculturing

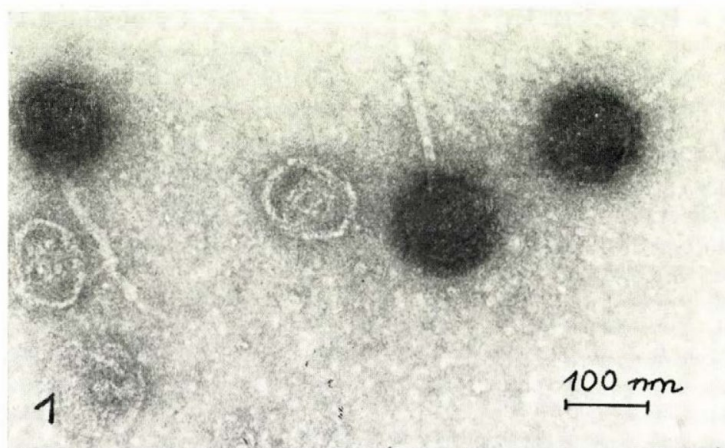


Fig. 1. Electron microscopic picture of phage *wx23*

of the plaques of phages *wx23* and *wx26* gave few plaque forming units. The plaques in soft agar were scraped off, suspended in culture medium and shaken vigorously by hand for 5 min. The extract was tested on the homologous *cin*⁻ culture for the released number of plaque formers. The number of p.f.u. per plaque was 4×10^5 for *wx23* and 10^5 for *wx26*.

Electron microscopy. The lysate of *B. cereus* strains 23 and 26, tested on homologous *cin*⁻ cultures, showed the presence of plaque formers. Electron micrographs (Figs 1 and 2) prepared from the concentrates of the two phages revealed B-type phages according to BRADLEY's classification [13]. The size of phages *wx23* and *wx26* is shown in Table I. The phages were similar in morphology to the converting phage and its clear mutant of *B. cereus* W, but they differed somewhat from the latter in size (the head of *wxc* is 73 nm in diameter and the length of its tail is 153 nm [4]). In the concentrates of phages *wx23* and *wx26* a number of tailless empty phage heads were seen (Fig. 1). This observation and results showing the low ratio of plaque formers in the phage

population, are in correlation and indicate a low degree of tolerance of the phages to mechanical injury.

In the concentrated lysate of the remaining three *B. cereus* strains 22, 27 and 28, repeated electron microscopic examinations failed to detect intact or defective phage structures. This was in agreement with the failure of bio-

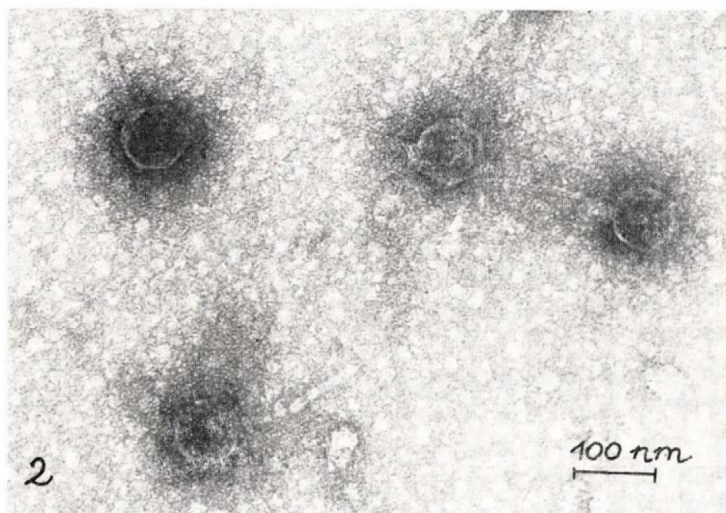


Fig. 2. Electron microscopic picture of phage *wx26*

Table I

Size of the structural elements
of phages *wx23* and *wx26*

Structural element, nm	<i>wx23</i>	<i>wx26</i>
Diameter of head	100	64
Length of tail	210	160

logical tests to show plaque formers on homologous *cin*⁻ strains and with the fact that lysates of these strains caused no lysogenic conversion.

Converting capacity of the lysates of B. cereus cultures. As shown in Table II, all *cin*⁻ derivatives including that of *B. cereus* W, were tested against all lysates. Plaque formation and conversion were highly strain-specific in case of phages *wx* and *wx23*. Phage *wx26* exhibited a less specific behaviour as it

Table II
Characteristics of crude lysates of B. cereus strains

<i>B. cereus</i> strain	Bacteriocin U/ml*	Phage p.f.u./ml*	Plaque production and lysogenic conversion by crude lysates of the cured strains					
			22 <i>cin</i> ⁻	23 <i>cin</i> ⁻	26 <i>cin</i> ⁻	27 <i>cin</i> ⁻	28 <i>cin</i> ⁻	W <i>cin</i> ⁻
22	800	0	—	—	—	—	—	—
23	2000	3 × 10 ⁵	—	p.c.	—	—	—	—
26	2000	8 × 10 ⁵	p.c.	—	p.c.	—	p.c.	—
27	3200	0	—	—	—	—	—	—
28	2000	0	—	—	—	—	—	—
W	3200	2 × 10 ⁵	—	—	—	—	—	p.c.

* Lysate of individual strains grown in BCM + yeast extract medium. Intensive gyration (in Gyrotory Shaker, New Brunswick, 350 r.p.m.) in the presence of 0.5 µg/ml of MC

p. = plaque formation on individual *cin*⁻ strains

c. = conversion of *cin*⁻ strains

— = neither plaque formation on, nor lysogenic conversion of, the cured strains

0 = no plaque formation on homologous *cin*⁻ strain

produced plaques on, and conversion of, not only the homologous but also two other *cin*⁻ bacteria.

The time course of conversion by phages *wx23* and *wx26* is seen in Table III. Homologous *cin*⁻ bacteria in the exponential phase of growth were infected with 5 m.o.i. and the degree of conversion was determined at 22°C

Table III
Conversion rate (per cent) and number of colony formers (10⁷/ml) in PBY_{e1} medium in presence and absence of chloramphenicol
 m.o.i.: 5 per colony formers

System		Assay for	CA	Time in minutes at 22°C					
bacterium	phage			0	15	30	60	120	600
23 <i>cin</i> ⁻	<i>wx23</i>	conversion	—	0	8	30	40	90	100
			+	0	0	0	1	2	2
23 <i>cin</i> ⁻		colony formers	—	1.2	2.0	2.5	5.0	5.5	8.2
			+	1.0	1.2	1.3	1.0	1.5	1.2
26 <i>cin</i> ⁻	<i>wx26</i>	conversion	—	1	10	45	70	90	100
			+	0	0	0	2	2	2
26 <i>cin</i> ⁻		colony formers	—	1.2	1.5	2.0	2.5	4.2	8.0
			+	1.6	2.0	2.0	2.0	2.0	2.0

CA = chloramphenicol: — nil, + 250 µg/ml

in the presence and absence of chloramphenicol. The number of colony forming units was determined in parallel experiments. Exponential phase bacteria showed 100% conversion in 5 hr, whereas with chloramphenicol the conversion rate was only 1–2%. On the basis of experiments with phage *wx* [7] it was evident that phages *wx23* and *wx26* converted the homologous *cin*⁻ bacteria more rapidly than did *wx* its *cin*⁻ culture. In the presence of chloramphenicol none of these phages converted the cultures to phospholipase A production (Table III). In agreement with previous findings [7], it would appear that the receptor for the two phages, similarly as the receptor for *wx*, is produced only in actively growing bacteria and no adsorption occurs in resting cells.

Examination of concentrated lysates of B. cereus strains and demonstration of the identity of bacteriocins with phospholipase A. The concentrates contained 10 000–60 000 units of bacteriocin (megacin A). As seen in Table IV, the degree of concentration varied from strain to strain.

Table IV shows the phospholipase A content and the specific activity of the concentrates. All concentrates were subjected to thin layer chromatography in order to confirm the presence of phospholipase A. Fig. 3 shows the cleavage of the concentrated lysate of *B. cereus* strain 27 on phosphatidyl ethanolamine. As lysophosphatidyl ethanolamine was not available, the lysate

Table IV

Bacteriocin ("megacin A") and phospholipase A activity of concentrates obtained from lysates of B. cereus strains

Strain	Protein, mg/ml	Megacin A titre, U/ml	Specific megacin A activity	Phospholipase A activity, U/ml	Specific enzyme activity
22	1.62	10 000	6 300	480	295
23	1.30	10 000	7 700	440	338
26	2.75	30 000	11 000	1440	525
27	4.10	60 000	14 500	2120	515
28	1.00	20 000	20 000	600	600
W	3.74	60 000	16 000	1440	385

from *B. cereus* W was used as a control of phospholipase A activity. The concentrated lysates of the five strains examined and of *B. cereus* W were similar in the order of specific activity for phospholipase A.

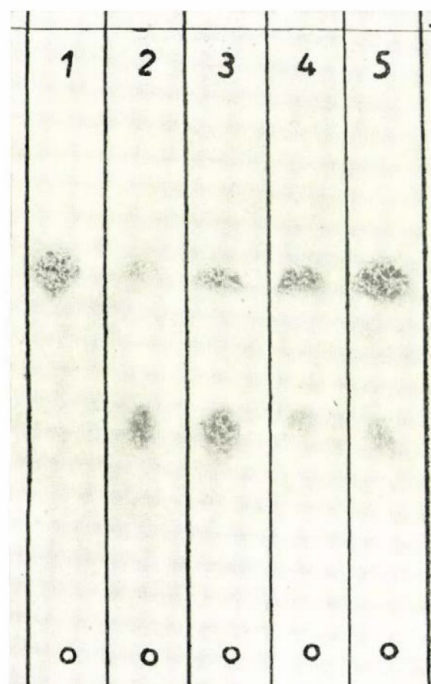


Fig. 3. Thin layer chromatography for identification of phospholipase A activity of the concentrates. Solvent, chloroform : methanol : water (65 : 25 : 4 v/v). Detection: iodine vapour followed by starch solution treatment of the silica gel layer. 1. L-phosphatidyl ethanolamine (dipalmitoyl). 2. Action of concentrate on *B. cereus* W strain. 3-5. Serial twofold dilution was made of the basal dilution of the concentrate obtained from strain 27. Lower spots indicate lyso-phosphatidyl ethanolamine

Discussion

Part of several dozen of *B. cereus* strains isolated from soil produced megacin A (phospholipase A). Five strains showing the presence of high titre bacteriocins in their lysates after MC induction were selected for further studies. These cultures exhibited multiple lysogenicity: in addition to the prophage responsible for megacin A production they carried another prophage differing from phage β of *B. cereus* W. Under the effect of ethidium bromide, all five strains gave off segregants not producing phospholipase A. In the lysates of three of the strains, phages converting to phospholipase A production could not be detected; accordingly, it may be assumed that, although the gene segment giving information for phospholipase A production was present, the prophage was defective in these cultures. The phages shown in the other two strains differed in size from converting phages of *B. cereus* W; their relationship to the latter, however, was proved by a similar antigenic

structure and high host specificity. It is therefore justified to regard *B. cereus* phages associated with phospholipase A production as a characteristic group of bacterial viruses and to use the term *wx* for their designation. Phages belonging to this group may differ in size and thermosensitivity. While the virions of the phages showed an identical degree of heat tolerance, phages *wx23* and *wx26* formed plaques only at temperatures lower than 37°C (optimum at 26°C). The relationship of the converting phages isolated in our studies is evident also from the fact that in resting cells the receptors for them are absent, whereas in dividing cells an unidentified precursor serves as the receptor. This hypothetical precursor loses its adsorbing capacity after incorporation into the cell.

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LEUKOCYTE MIGRATION INHIBITION BY A SPECIFIC ANTIGEN IN HUMAN TYPHOID FEVER

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Summary. Cellular reactivity to heat-killed *Salmonella typhi* antigen was investigated by the leukocyte migration inhibition (LMI) method in 33 *S. typhi* infected patients and in 32 control persons. In the typhoid group a statistically significant LMI was observed as compared to the members of the control group. A correlation was found between the level of the cellular sensitivity and the time elapsed between onset of the disease and performance of the test. Previous typhoid vaccination had no influence on the LMI. No correlation was found between the agglutinin titres and the sensitivity demonstrated by the LMI test. The value of the method in studies of cellular immunity in typhoid fever is discussed.

Cell-mediated immune response in typhoid fever has scarcely been studied. BASHIROVA [1] using *Salmonella typhi* antigen observed a blastic transformation of lymphocytes in typhoid patients. SVEHAG and BOKLUND [2] found a migration inhibition of leukocytes by *S. typhi* glycoprotein in persons immunized with killed *S. typhi*. KUMAR *et al.* [3] examined 22 typhoid patients with leukocyte migration inhibition (LMI) method. Since the LMI method of SØBORG and BENDIXEN [4] had enabled us to detect cellular immunity in brucellosis [5], we chose the same method for studying the cellular immunity of *S. typhi* infected patients.

Materials and methods

Patients. Thirty-three hospitalized patients infected during a small typhoid epidemic were tested. *S. typhi* infection was verified by bacteriological and serological methods. Out of the 33 patients 27 had clinical symptoms while 6 had no clinical disease; they were admitted because of positive blood cultures or coprocultures and/or high agglutinin titres found in a screening test. Sixteen patients had been vaccinated against typhoid fever 1 to 2 months previously. By the time of LMI testing, 28 patients were bacteriologically negative and only 2 had clinical symptoms. The period between the onset of the disease or the positive laboratory findings (in the case of symptom-free patients) and the time of LMI testing varied from 3 to 61 days with a mean of 39.1 days. All the LMI tests were performed within a three-day period. In the case of 17 patients a second test was made six months later.

Thirty-two 6 to 14 year old children with non-enteric infection (scarlet fever, upper respiratory disease, mumps) and with no history of typhoid fever or typhoid vaccination served as controls.

Laboratory methods. LMI tests were carried out by the method of SØBORG and BENDIXEN [4]. As antigen a heat killed *S. typhi* culture of strain 10 086 (Hungarian National

Collection of Medical Bacteria, Budapest) was used. The leukocytes of each person were tested by two antigen concentrations i.e. 50×10^6 and 10×10^6 bacteria per ml. Both control and antigen chambers were prepared in triplicate. Migration indices were calculated by dividing the average migration areas in the antigen chambers by those in the control chambers. A difference of more than 20% in the means was found significant when evaluated by Student's *t* test. Therefore LMI tests with a migration index less than 0.8 were considered positive.

At the time of LMI testing the patients were tested for specific agglutinin titres by the usual tube agglutination method as well.

Results

Individual migration indices calculated from tests carried out with 50×10^6 and 10×10^6 bacteria per ml are presented in Fig. 1 and Fig. 2, respectively. Means and standard deviations of the migration indices are summarized in Table I.

LMI tests performed with the higher antigen concentration were positive in 25 out of 33 typhoid patients and in 7 out 32 controls. With the lower antigen concentration the rate of positivity was 16/33 in the typhoid group and 3/32 in the control group. Differences in the occurrence of positive tests

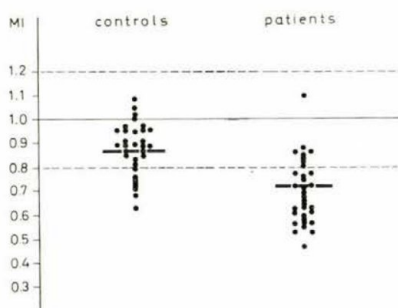


Fig. 1. Leukocyte migration indices of control persons and typhoid patients (antigen: 50×10^6 /ml heat killed *S. typhi*)

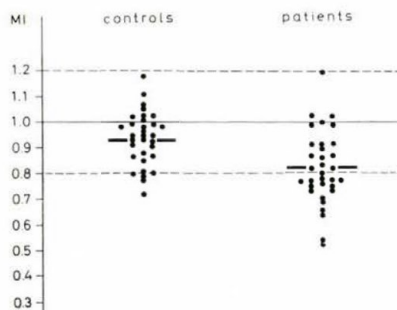


Fig. 2. Leukocyte migration indices of control persons and typhoid patients (antigen: 10×10^6 /ml heat killed *S. typhi*)

Table I

LMI indices in typhoid patients and in control persons

Antigen concentration ($\times 10^6$ bacteria/ml)	LMI index (mean $\pm$ SD)	
	Typhoid group	Control group
50	0.72 ± 0.13	0.87 ± 0.10
10	0.82 ± 0.14	0.93 ± 0.10

between the typhoid and the control group were found significant for each antigen concentration when tested by the χ^2 test (for the higher concentration: $\chi^2 = 17.1$; $p < 0.001$; for the lower concentration: $\chi^2 = 11.9$; $p < 0.001$). Similarly significant differences were found between the control and the patient group when comparing the means of the migration indices with Student's t test (for the higher antigen concentration $t_{63} = 5.0$, $p < 0.001$; for the lower concentration, $t_{63} = 3.66$, $p < 0.001$).

According to the reactivity to the different antigen concentrations, the patients could be divided into three groups (Table II). In the first group LMI tests were positive with both antigen concentrations, in the second group the tests were positive with the higher antigen concentration only, while in the third group there was no inhibition by any of the concentrations used. Of the six symptom-free patients, three belonged to the first and three to the third group.

Table II

Correlation between LMI results and the interval between onset of the disease and LMI testing

	Distribution of patients according to the results with 50×10^6 and 10×10^6 bacteria per ml		
	positive with both concentrations	positive with 50×10^6 negative with 10×10^6	negative with both concentrations
	16	9	8
Interval (days) between onset of disease and LMI testing (mean and range)	44.4 (30-61)	34.3 (15-57)	26.4 (3-61)

Some correlation was observed between the results of LMI and the interval between onset of the disease and the time of testing; this period was longest in the first group and shortest in the third one (Table II).

Table III shows that the distribution of the agglutinin titres was about the same in all the groups.

Table III
Correlation between the results of the LMI test and agglutinin titres

Agglutinin titre	Result of LMI test with 50×10^6 and 10×10^6 bacteria per ml					
	positive with both concentrations		positive with 50×10^6 negative with 10×10^6		negative with both concentrations	
	O	H	O	H	O	H
Negative	5	6	5	5	3	1
1 : 100	4	2	1	2	2	1
1 : 200	4	1	1	2	2	3
1 : 400	3	4	2		1	2
1 : 800		2				1
1 : 1600		1				
Number of patients tested	16		9		8	

LMI tests were repeated in 17 patients six months later. Results of the first and the second tests are compared in Table IV. Sensitivity remained practically unchanged in those who had been positive in the first tests. Moreover in four out of five patients who had been LMI negative with both antigen concentrations, sensitivity developed by the time of the second test.

Table IV
Comparison of the results of LMI tests repeated at six months interval

Second test	First test			Total
	positive with both concentrations	positive with 50×10^6 bacteria/ml negative with 10×10^6 bacteria/ml	negative with both concentrations	
Positive with both concentrations	7	2	1	10
Positive with 50×10^6 bacterial/ml Negative with 10×10^6 bacteria/ml	2	1	3	6
Negative with both concentrations	—	—	1	1
Total	9	3	5	17

Discussion

Our results are comparable to those of KUMAR *et al.* [3] in that the LMI test demonstrated a cellular reactivity to heat killed *S. typhi* in persons with

S. typhi infection. This reactivity may develop in the course of clinical disease and after latent infection as well.

Reactivity demonstrated by the LMI test was found to take more time to develop than humoral immunity generally does. This was indicated by two observations. First, sensitivity to both antigen concentrations could be demonstrated only in cases where at least 30 days had elapsed between the onset of the disease and the performance of the LMI test (Table II). Second, 4 out of 5 patients who had been negative during the disease or immediately after it, proved positive when tested six months later (Table IV). The situation is similar in human brucellosis [5].

The lack of correlation between LMI positivity and the agglutinin titres speaks for the fact that the reactivity demonstrated by the LMI test is independent from the presence of specific antibodies in the serum.

Though LMI seems to be a useful method in studying cellular immunity in typhoid fever, its results should be considered with certain reservations. Like KUMAR *et al.* [3] we also found some LMI positive persons in the control group, although this group comprised subjects with no known history of typhoid fever or typhoid vaccination. On the other hand some typhoid patients proved negative in the test. The reliability of the test could not be improved by increasing or decreasing the concentration of antigen. A change in the antigen concentration changed the positive/negative ratio at almost the same rate in both the patient and the control groups. We have no reason to suppose that a positive LMI in a control patient is aspecific since it may well be due to a previous unnoticed infection with *S. typhi* or rather with an antigenically related salmonella. On the other hand, a negative LMI in a typhoid patient may be explained by the fact that the test was performed before cellular reactivity could have developed.

Thus, individual LMI results are of no diagnostic value in typhoid fever. However, average migration index values of groups may give useful informations on the role of cellular immunity in the pathogenesis of the disease.

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NOTE

INCIDENCE OF NIACIN NEGATIVE MYCOBACTERIUM TUBERCULOSIS STRAINS

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(Received May 21, 1975)

Summary. Two niacin negative *Mycobacterium tuberculosis* strains were isolated from a patient. Other *M. tuberculosis* strains isolated from the same patient were niacin positive.

Although niacin positivity is one of the most reliable characteristics of *Mycobacterium tuberculosis* [1], strains failing to produce this substance may be encountered. TSUKAMURA described a total of 13 niacin negative cultures [2]. Since 1973 we have been testing all acid fast isolates for the niacin reaction [3]. Examination of 2094 isolates including 1723 *M. tuberculosis* strains, revealed niacin negative cultures distributed as follows: 2 *M. tuberculosis* (from 1 patient), 12 *M. bovis* (from 2 patients), 5 *M. bovis* BCG and 65 atypical mycobacteria, nocardias, etc. (rhodochrous complex).

The 2 *M. tuberculosis* strains remained niacin negative throughout a series of subcultures. Eight strains from the same patient were negative at first examination, but became positive on repeated testing. Five negative isolates failed to grow on subculturing. The two strains with persistent niacin negativity were characterized by a slow catalase reaction, nitrate reductase production and failure to hydrolyse Tween 80. They were arylsulphatase negative and phosphatase positive. In the Bönicke series they gave a strong reaction with substrate 3 and a weak reaction with substrates 5 and 6. In liquid medium the cultures showed cord formation. The result of drug sensitivity testing performed by estimating the proportion of resistant colonies [4] was as follows: isoniazid, streptomycin, etambutol resistant; viomycin, cycloserine, ethionamide and rifampicin sensitive. In view of the present result and of TSUKAMURA's data, whose niacin negative strains were resistant to 5 different drugs, resistance seems to be associated not only with catalase activity, pathogenicity and iron accumulation, but also with niacin production.

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USE OF ELUTION MARKER FOR THE INTRATYPIC CHARACTERIZATION OF POLIOVIRUS STRAINS

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Public Health Station, Szeged

(Received April 2, 1975)

Summary. Elution marker was used for intratypic characterization of poliovirus strains with $\text{Al}(\text{OH})_3$ gel as adsorbent. The virion suspensions to be tested were partially purified by chromatography and labelled with ^{32}P . In the labelled preparations of wild virus strains practically all radioactivity was found in virus-specific bond, whereas in those of the vaccine strains and isolates of vaccine origin a considerable, but variable, proportion of the activity was bound to residual cell components. For this reason, the EC_{50} value (i.e., the phosphate molarity corresponding to the 50% adsorption equilibrium of virions), for the vaccine and vaccine-like strains showed a wide scattering. The activity bound to cell components was removable from the gel with 0.005 M phosphate buffer, whereas the elution maxima for all the poliovirus strains examined so far were over 0.02 M. The elution marker was calculated for 25 type-2 and 30 type-3 strains, all isolated during or soon after vaccination periods from cases suspect of poliomyelitis, both with and without taking into account the cell-bound activity. In the latter case, the EC_{50} values agreed with that for the corresponding reference vaccine strain much better than in the former; furthermore, the type-2 reference vaccine strain and the type-2 isolates, indistinguishable from the wild reference strain by the original method, could easily be differentiated on the basis of the corrected EC_{50} value. The possibility that the lack of an appreciable proportion of cell-bound radioactivity may reflect the pathogenicity of poliovirus strains is discussed.

Numerous *in vitro* methods have been developed for intratypic differentiation of poliovirus strains [1–7]. These tests are of great importance in revealing the aetiology of poliomyelitis-like cases occurring during or soon after vaccination periods. We have introduced the $\text{Al}(\text{OH})_3$ gel-adsorption procedure described by THOMSEN *et al.* [8, 9]. By its use numerous strains isolated in Hungary [10] and abroad [11, 12] have been characterized.

The vaccine-like poliovirus isolates, first of all those of the type-3 virus, usually showed a weaker gel adsorption than did the homotypic reference vaccine strain. Even the readiness to adsorption of the latter strain tended to decline while the strain was serially passaged in cell cultures. Furthermore, there were difficulties in differentiating type-2 vaccine-like strains from the wild reference strain.

The present work was undertaken to eliminate some sources of error from the procedure.

Materials and methods

Poliovirus reference strains. Type-1 strains: Mahoney and LSc 2ab. Type-2 strains: 5148-II and P712 Ch2ab. Type-3 strains: 1072/61 Freiburg, 1658/63 Freiburg and 367/60-III (wild strains) and Leon 12a,b (vaccine strain). Strains 5148-II and 367/60-III originated

from the Department of Virology, National Institute of Hygiene, Budapest. For the origin of the other strains, see [10].

Poliovirus isolates. Twenty-five type-2 and 30 type-3 strains were isolated during or soon after vaccination periods in different countries.

Cell cultures. Rhesus monkey primary kidney cell cultures as well as HEp-2, F1 and HeLa cell cultures were used.

Titration of virus infectivity. Before titration, the fractions eluted from the $\text{Al}(\text{OH})_3$ gel were dialysed against a mixture of equal volumes of distilled water and Hanks' solution. Tenfold dilution series were prepared and five primary monkey kidney cell cultures were inoculated with 0.1 ml portions of each dilution. The titre was calculated on the basis of the reading on the 5th day postinoculation.

We have developed a rapid test for comparing infectivity values; *viz.*, 0.2 ml samples of each dialysed fraction were transferred into five F1 cell cultures each and the results were read after an incubation period of 15–17 hr. The fraction exerting the most pronounced cytopathic effect was regarded as the elution maximum of the virus.

Elution test. (1) *Partial purification of the virion suspension.* Essentially the method evolved by THOMSEN *et al.* [8, 9] was used. Cell cultures (primary monkey kidney, HEp-2 or F1 cultures) were labelled with ^{32}P ($\text{Na}_2\text{H}^{32}\text{PO}_4$) and infected with the virus strain under study. As the cells had been destroyed, the cultures were frozen and thawed several times and centrifuged at 5000 rpm for 10 min. The pH of the supernatant was adjusted to 5.5–6.0 with 1.0 M acetate buffer pH 5.5, and 1.0 ml 0.25% $\text{Al}(\text{OH})_3$ gel suspension (C₇, Behring-Werke) was added to each supernatant. Subsequently, the adsorbent was washed five or six times, by sedimentation and decantation, with 0.1 M acetate buffer pH 5.5. Elution was performed with 0.5 M phosphate buffer pH 7.5. The volume added was equal to the initial volume of the supernatant. The eluates were dialysed at 4 °C against a mixture of equal volumes of Hanks' solution and distilled water and centrifuged for 20 min at 15 000 rpm. A 5-ml sample of the supernatant was fractionated on a DEAE or ECTEOLA cellulose column (6 × 7 cm). The first 2-ml sample was designated E₀, the subsequent 3-ml, E₁. Fractionation was continued with 9 ml 0.02 M phosphate buffer and three consecutive fractions (E₂, E₃ and E₄), each 3 ml in volume, were collected. In the elution test, the eluate of the highest activity (usually the E₃ fraction) was used.

(2) *Performance of the test.* (a) *Successive elution test.* Of the DEAE cellulose eluate chosen for the test, a 5-fold diluted sample was transferred into isotonic NaCl solution adjusted to pH 7.5 with Tris buffer. Then a 5-ml amount of this was transferred onto 1 ml 1% $\text{Al}(\text{OH})_3$ gel and, after 15 min adsorption at room temperature, successive elution was performed with the following molarity gradients of the phosphate buffer: 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.24 and 0.32. As diluent Tris buffer was used up to 0.08 molarity and distilled water further on; 2-ml doses were added throughout. Elution time was 20 min. The height of elution peak was expressed in per cent of the whole eluted activity.

(b) *Parallel elution test.* A one-ml sample of 0.25% $\text{Al}(\text{OH})_3$ gel was measured into each of nine centrifuge tubes and 2 ml of the partially purified virus suspension was added to each tube. The virus suspension had been diluted with 0.1 M acetate buffer pH 5.5 to an activity producing 2000 cpm/ml. If possible, the dilution was higher than 1 : 10. After a period of adsorption, the supernatants were poured off and, to elute the virus, 2 ml of Tris buffer was added to the first tube and 2 ml phosphate buffer to each of the others. After thorough shaking, elution was performed at room temperature for 20 min. To express the eluted activity in per cent, the activity eluted with 0.32 M phosphate buffer was taken as 100%. On this basis, a cumulative curve was drawn on which the phosphate buffer molarity bringing about the 50% adsorption equilibrium was regarded as the EC_{50} value for the strain under study. Derivation of the cumulative curve resulted in practically the same elution peaks as obtained by the successive elution method. The activity of the elution peaks thus obtained was expressed in per cent of the total eluted activity.

Results

Searching for the cause of the high spreading of elution markers, we examined the elution of radio-labelled non-infected cell culture fluids as described above for virus-containing culture fluids. In the DEAE cellulose fractions the distribution of radioactivity was found to be similar as that for the purified fluids of cultures infected with attenuated or wild poliovirus (Table I). The

Table I

Distribution of radioactivity in DEAE column-chromatographic fractions of extracts of non-infected and virus-infected cell cultures

Cell culture	Virus strain	Activity (cpm/0.1 ml) of DEAE column-chromatographic fractions				
		E ₀	E ₁	E ₂	E ₃	E ₄
Primary monkey kidney	—	16	101	2505	314	215
HeLa	—	14	102	2307	2062	505
F1	—	18	316	2368	1068	422
HEp-2	—	18	402	3170	1320	502
Primary monkey kidney	Leon 12a ₁ b	2	347	7126	724	204
F1	LSc 2ab	2	928	2202	402	99
HEp-2	P712 Ch2ab	22	1160	3820	828	206
Primary monkey kidney	367/60-III	28	800	2802	212	122
F1	5148-II	24	1279	2392	2202	315
HEp-2	Mahoney	28	1664	7122	1629	374

differences between the values in different rows are mainly due to quantitative differences in labelling.

In further experiments, starting from non-infected cell cultures, we adsorbed to $\text{Al}(\text{OH})_3$ gel the DEAE cellulose fraction showing peak activity. Then the successive elution test was performed. These experiments were carried out with HEp-2, HeLa and/or F1 culture fluids harvested after 72 hr incubation as well as with culture fluids harvested from monkey kidney primary cell cultures as the monolayers had been completed (Fig. 1). The elution peak appeared at 0.005 M phosphate buffer in each case. (Starting from older cultures resulted in a second peak at 0.16 M buffer.)

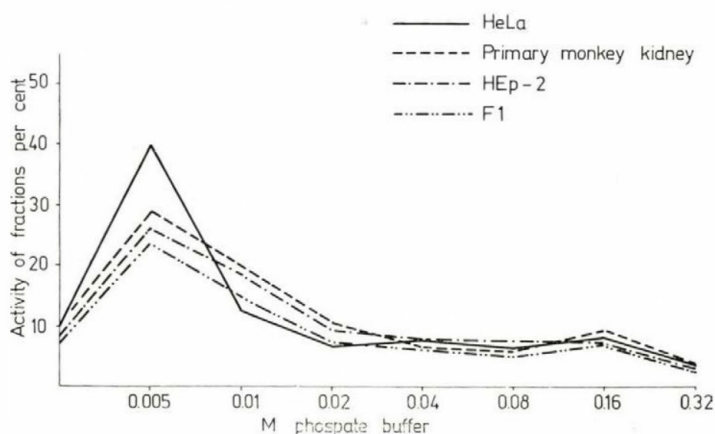


Fig. 1. Fractionation on $\text{Al}(\text{OH})_3$ gel of ^{32}P -labelled partially purified non-infected cell culture preparations. (Successive elution tests)

Next, parallel elution tests were performed with culture fluids harvested from F1 cultures infected with different viruses. The EC_{50} values were determined and the elution activity peaks were calculated by deriving the cumulative curves. The same viruses were tested in successive elution experiments. Results are shown in Fig. 2.

The derived cumulative curves for the vaccine strains (LSc 2ab, P712 Ch2ab, Leon 12a₁b) showed two peaks each, whereas only one, well-defined, peak was displayed by the wild reference strains (Mahoney, 5148-II and 367/60-III). According to the results of infectivity titrations (successive tests), the elution maximum for the strains LSc 2ab, P712 Ch2ab and Leon 12a₁b was at 0.32, 0.16 and 0.16, respectively. The corresponding peaks for the type-1, type-2 and type-3 wild reference strains were at 0.02–0.04, 0.08 and 0.32 M phosphate buffer, respectively. The first (lower) peak for the vaccine strains appeared at 0.005 molarity, *i.e.*, it coincided with the maximum for non-infected cell cultures. At the same molarity, infectivity titration revealed no or very little virus.

Based on these experiences, we plotted the cumulative activity curves omitting the non-viral activity eluted below 0.02 molarity and calculated the EC_{50} values from these curves. To distinguish these values from the original EC_{50} , we introduced the designations $EC_{50(v)}$ and $EC_{50(t+v)}$ where v stands for virus-bound activity and t for tissue-bound activity. In the case of the vaccine strains there was an appreciable difference between $EC_{50(t+v)}$ and $EC_{50(v)}$ the respective values being 0.24 and 0.28 for LSc 2ab; 0.065 and 0.112 for P712 Ch2ab; and 0.059 and 0.0815 for Leon 12a₁b. As to the wild reference strains, the $EC_{50(v)}$ approximately coincided with the corresponding $EC_{50(t+v)}$ except for strain 5148-II, where some difference was found between the two values ($EC_{50(v)} = 0.054$, $EC_{50(t+v)} = 0.051$). There was no reason to illustrate the cumulative curves for the remaining two wild reference strains.

We performed elution tests with re-dialysed samples of the $Al(OH)_3$ gel fractions showing the highest virus-specific activities, to see how the EC_{50} values obtained in this way agreed with the calculated values. The experiments were carried out in several parallel tests with the vaccine strains and the three wild reference strains. Mean values and standard deviations are shown in Table II.

It is seen that the $EC_{50(v)}$ values obtained with the virion suspensions separated from the tissue elements agreed well with the calculated $EC_{50(v)}$ values. We performed this test with the peak fractions of the wild Freiburg strains 1072/61 and 1658/63 as well, to compare the results with those obtained for the homotypic wild reference strain 367/60-III. The agreement was good between strains 1072/61 and 367/60-III, but strain 1658/63 was somewhat different. In this case, however, the DEAE cellulose fraction of maximum activity contained considerable amounts of cellular elements.

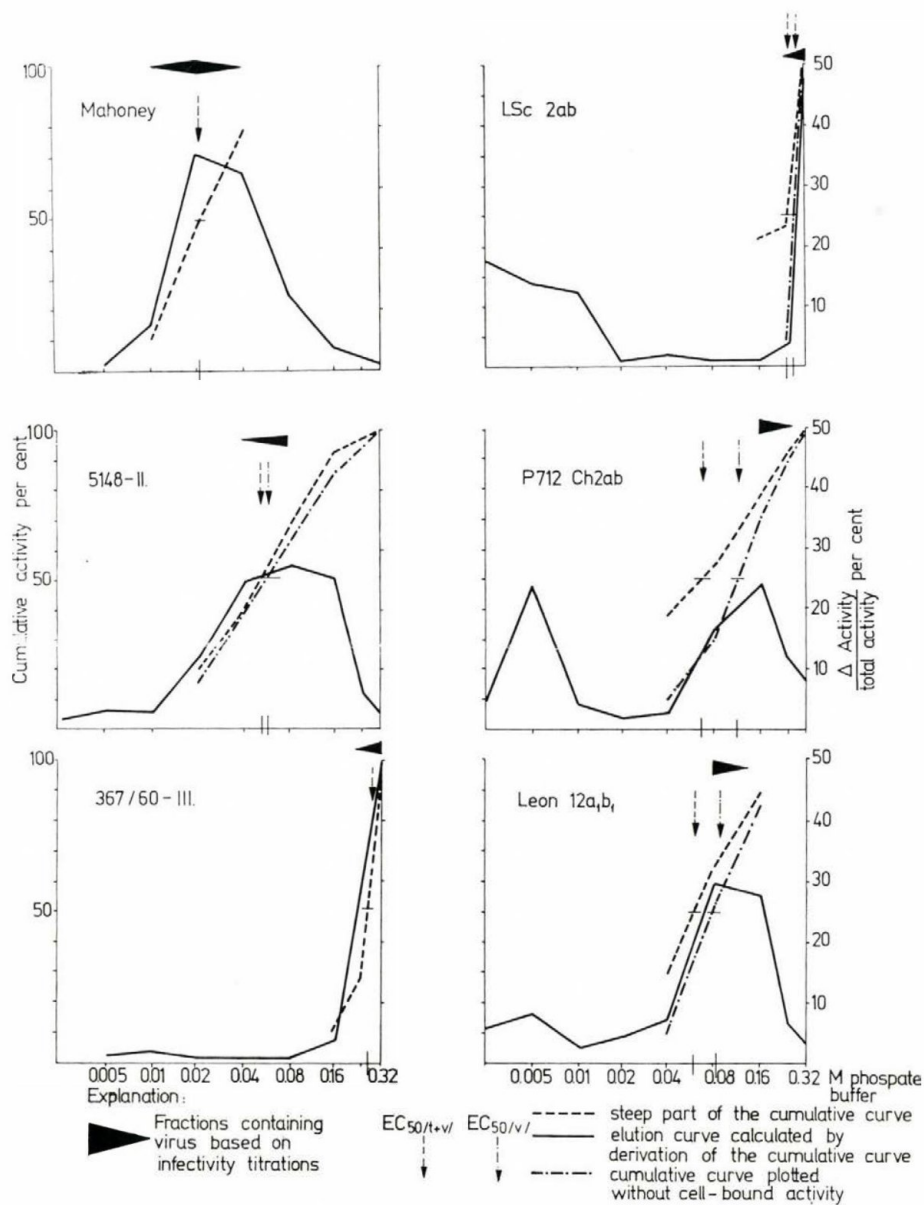


Fig. 2. Fractionation on $\text{Al}(\text{OH})_3$ gel of partially purified reference poliovirus strains propagated in F1 cell cultures and labelled with ^{32}P

Table II

$EC_{50(v)}$ values for poliovirus reference strains obtained with the virus maximum fractions eluted from $Al(OH)_3$ gel

Virus		$EC_{50(v)}$	
Strain	Type	$\bar{x}$	s
Mahoney	1	0.02*	—
LSc 2ab	1	0.27	0.017
5148-II	2	0.06	0.0083
P712 Ch2ab	2	0.137	0.036
367/60-III	3	0.25	0.01
Leon 12a ₁ b	3	0.094	0.014

* M phosphate buffer

Finally, the $EC_{50(v)}$ values calculated for the 25 type-2 and 30 type-3 poliovirus strains isolated from cases suspect of poliomyelitis during or after vaccination periods were compared with the $EC_{50(t+v)}$ values calculated for the same strains (Figs 3 and 4). We selected these isolates from those which had been found to have elution properties consistent with, moderately different from, or considerably different from, the elution markers published in literature.

It was found that the $EC_{50(v)}$ values for both the type-2 (Fig. 3) and type-3 strains (Fig. 4) spread in a considerably narrower range than did the

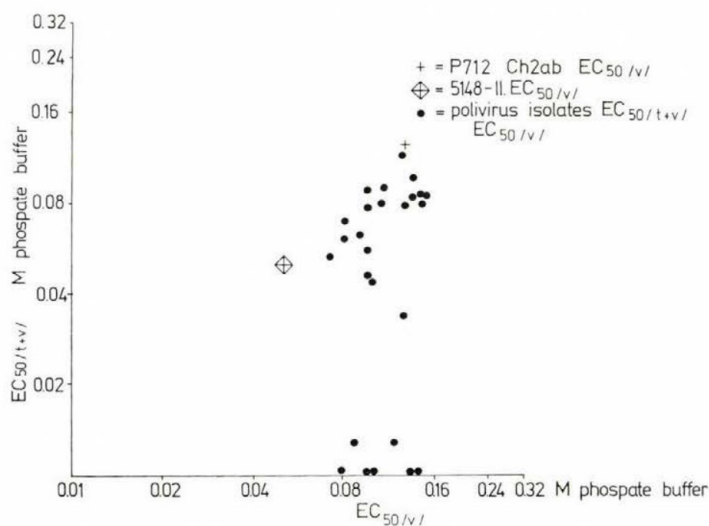


Fig. 3. Comparison of the $EC_{50(t+v)}$ and $EC_{50(v)}$ values for type-2 poliovirus strains

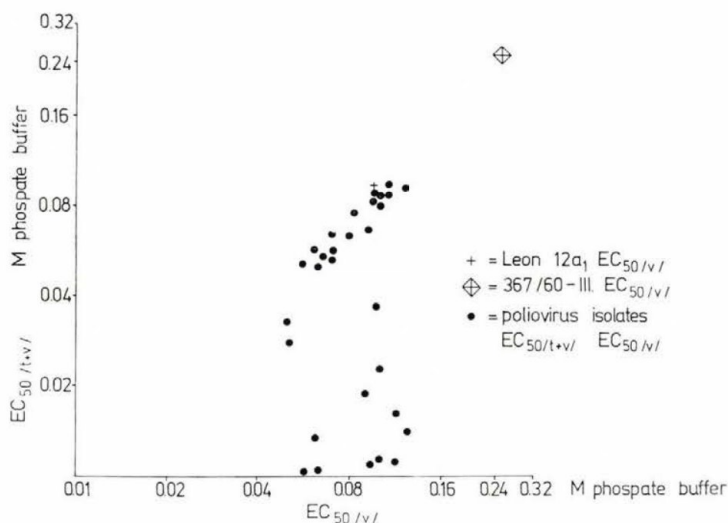


Fig. 4. Comparison of the $EC_{50(t+v)}$ and $EC_{50(v)}$ values for type-3 poliovirus strains

$EC_{50(t+v)}$ values. On the basis of the $EC_{50(v)}$ values the vaccine strains as well as the isolates are well-distinguishable from the respective wild reference strain, and even the vaccine-like type-2 strains can be differentiated from the homotypic wild strain.

Discussion

In previous studies, the elution test with $Al(OH)_3$ gel as adsorbent proved suitable for the differentiation from the homotypic reference strain of type-1 and type-3 vaccine strains and isolates of vaccine origin. As to the type-1 strains, the EC_{50} value for the Mahoney strain was much lower than for the vaccine and vaccine-like strains so that the presence of readily eluable cell-bound radioactivity in the latter materials hardly diminished the differences between the EC_{50} values.

The wild reference strain for type-3 poliovirus has a rather high EC_{50} value, higher than that for the homotypic vaccine strain. The difference is enhanced by the presence of cell-bound activity in the semi-purified virion suspensions of vaccine-like strains.

As to the type-2 strains, we had failed to ensure intratypic differentiation by the $Al(OH)_3$ elution test alone, because the difference existing between the $EC_{50(v)}$ values was reduced to an insignificant level by the presence of cell-bound activity. Differentiation only became possible when the cell-bound activity was omitted from the calculation of EC_{50} values.

It is advisable, therefore, to apply the successive elution test in intratypic differentiation of poliovirus strains isolated from patients. If the parallel test is used, the cumulative curve should be derived, the eluted peak fractions separated and the activity of the first peak omitted from the calculation of EC_{50} . In this way it can be proved if the difference between the EC_{50} s is due to differences in the adsorptive properties of the virions.

Strict observation of the standard experimental prescriptions still allows for differences in the experimental conditions, e.g., the cell culture may or may not carry viruses, or another virus excreted by the patient may be co-passaged with the poliovirus under study. The presence of such agents may influence the poliovirus-host relationship.

Radioactive labelling is in the first place of practical importance, since it accelerates the demonstration of the presence and of the concentration of the virus. If another isotope is used instead of ^{32}P , the method of purification must be changed. The main requirements in purification are (a) that in the purified virus most of the radioactivity should be bound to the virions, and (b) that the adsorption and elution properties of the virions should not be affected by the purification procedure.

The presence or absence of considerable amounts of cell-bound radioactivity in partially purified virion preparations may reflect differences in the intracellular events occurring during the viral reproduction cycle. The data available are insufficient to confirm this assumption. Nevertheless, it seems reasonable to investigate in detail the assumed relationship between the residual cell-bound activity and the pathogenicity of the virus.

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THE EFFECT OF ANTI-LYMPHOCYTE AND ANTI-THYMOCYTE SERUM ON THE PATHOGENESIS OF RAUSCHER LEUKAEMIA IN INBRED MOUSE STRAINS

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Summary. Rauscher virus causes in inbred Balb/c mice a rapidly progressing hyperacute, in C57B1/10Sn mice an incipient, spontaneously healing leukaemia. In DBA/1 and DBA/2 mice the appearance of leukaemia is followed by a partial remission then by an exacerbation of the disease. The infection in C57B1/10Sn, DBA/1 and DBA/2 mice results in a significant tumour-specific immune response. Inhibition of the immune response is followed by an increased progression of leukaemia in DBA/1 and DBA/2 mice only. It is assumed that in C57B1/10Sn mice the remission of incipient leukaemia is associated with a resistance determined in the target cells, whereas in DBA/1 and DBA/2 mice the remission is due to a tumour-specific immune response. As in animals treated with anti-lymphocyte and anti-thymocyte serum the course of the disease runs proportionally to the degree of the inhibition of the immune response, the tumour-specific antibodies play a decisive role in the elimination of the tumour cells. In Balb/c, DBA/1 and DBA/2 mouse strains failing to exhibit a spontaneous reversion of the tumour cells, the appearance of a significant tumour-specific immune response depends on the resistance against the helper component of the Rauscher virus complex.

It has been shown that in mouse strains resistant to Rauscher leukaemia virus the infection is followed by the appearance of tumour-specific antibodies, whereas the immune response is absent in sensitive mice [1]. From studies on the heredity of susceptibility to Rauscher leukaemia we have concluded that remission in manifest leukaemia may depend on the resistance to the helper component [2] of the Rauscher virus complex [3]. Based on these findings, it seemed desirable to elucidate the role of cellular and humoral immune response and the correlation between the antibody-producing capacity and the susceptibility to the helper virus. To inhibit the tumour-specific immune response, anti-lymphocyte and anti-thymocyte serum were chosen, as according to SENSENBRENNER *et al.* [4], of the immunosuppressive agents anti-lymphocyte serum is the less injurious to haemopoietic cells. To ensure the effectiveness and selectivity of immunosuppressive action, the antisera were given prior to infection. Sensitivity of the inbred mouse strains to components of the Rauscher virus complex was examined by the method of AXELRAD and STEEVES [5] elaborated for Friend virus.

Materials and methods

Virus. The Rauscher virus was kindly supplied by Professor V. M. BERGOLTS (Gertsen Institute for Oncology, Moscow) and was maintained in adult Balb/c mice. The experimental animals were infected intraperitoneally with 0.2 ml cell-free supernatant of a 20% homogenate prepared with phosphate buffer saline from leukaemic spleens.

Mice. Inbred Balb/c and DBA/2 mice were purchased from the Institute for Breeding of Laboratory Animals, Gödöllő, Hungary; the inbred DBA/1 and C57B1/10Sn mice were obtained from the National Blood Transfusion Service, Budapest. In the experiments offsprings of these animals and their F1 hybrids were used; infection with Rauscher virus was performed at the age of 8 weeks. For the determination of spleen index, humoral and cellular immune response and of spleen focus-forming effect, at intervals 3 mice were sacrificed from each of the groups representing different dilutions of the virus.

Humoral immune response. Antibody content of serum samples taken from the retro-orbital sinus was determined by indirect immunofluorescence. In the first phase of the reaction, 2×10^6 leukaemic cells were incubated with 0.05 ml serum then to the washed cells 0.05 ml anti-mouse swine globulin (Sevac, Prague) was added. Antibody titre was expressed as fluorescence index. The method was described in detail earlier [1].

Cellular immune response. The ^{51}Cr release test was performed as described by OREN *et al.* [6]. Lymphocytes were obtained from the cervical, axillary, inguinal and retroperitoneal lymph nodes; the target cells originated from the spleen of leukaemic Balb/c mice. The cells were washed twice in Eagle's MEM medium (pH 7.3, Flow Laboratories Inc., Rockville, Md., U.S.A.) containing 10% heat-inactivated calf serum. Viability of the cells was checked by staining with 1% trypan blue; only suspensions showing less than 10% stained cells were used. The tumour cells were labelled with $\text{Na}_2^{51}\text{CrO}_4$ (Institute of Radioactive Isotopes, Hungarian Academy of Sciences, Budapest) exerting a specific activity higher than $10 \mu\text{Ci}/\mu\text{g}$ Cr until the end of the experiments. To 2×10^7 target cells $150 \mu\text{Ci}$ of ^{51}Cr were added and the mixture was incubated at 37°C for 40 min with gentle shaking at 5 min intervals. Finally, the labelled cells were washed in 10 ml MEM supplemented with calf serum. To 2.5×10^4 target cells 5×10^6 lymphocytes were added (200:1) and the mixture was incubated in 5% CO_2 atmosphere at 37°C for 4 hr. Cytotoxicity was expressed as percentage of the radioactivity of the supernatants and of the radioactivity released from 2.5×10^4 target cells after three subsequent freezings and thawings. The degree of spontaneous release of ^{51}Cr and the effect on normal Balb/c spleen cells of the lymphocytes of DBA/1, DBA/2 and C57B1/10Sn mice were used as control. Measurement of gamma radiation was performed with the NK-108 Gamma impulse analyser.

Preparation, assay and dosage of anti-lymphocyte and anti-thymocyte serum. Suspensions from the lymph nodes, spleen and thymus of random-bred Swiss mice were used for the immunization of rabbits as described by GRAY *et al.* [7]. Lymphocytes were injected in doses of 10^7 , 1.5×10^7 and 5×10^6 , thymocytes in doses of 2×10^7 , 10^7 and 2.5×10^6 cells/kg body weight; the first injection was given intravenously without adjuvant, the subsequent two ones intramuscularly with complete Freund adjuvant (Difco, Detroit, Mich., U.S.A.). The rabbits were bled on the 10th day after the last injection and the sera were adsorbed with homologous erythrocytes. The cytotoxic titres of the anti-lymphocyte serum pool and the anti-thymocyte serum were 1:8000 and 1:4000, respectively. The experimental animals received 0.2 ml doses of anti-lymphocyte serum and 0.4 ml doses of anti-thymocyte serum on the 15th, 11th, 7th and 3rd day prior to infection; 10 days after infection they were injected intraperitoneally with 0.5 ml anti-lymphocyte serum or with 2×0.5 ml anti-thymocyte serum.

Enumeration of spleen foci. The virus material obtained from the spleen of Rauscher leukaemic Balb/c mice was serially diluted from 10^{-1} to 10^{-6} . Balb/c, DBA/1, DBA/2, C57B1/10Sn and (Balb/c $\times$ C57B1/10Sn) F1 mice were injected intraperitoneally with 0.2 ml amounts of undiluted and diluted virus. Nine days after infection the spleens were removed and fixed in Bouin solution. Separate colonies of tumour cells appearing on the spleen surface were counted and the number of focus-forming units was calculated for one ml of the undiluted virus suspension [5].

Results

Course of disease and tumour-specific immune response in mice infected with Rauscher leukaemia virus. The degree of splenomegaly following the infection was expressed in spleen index (average weight of normal animals'

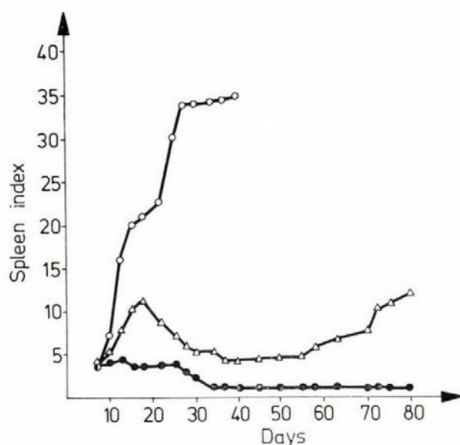


Fig. 1. Splenomegaly in mouse strains infected with Rauscher leukaemia virus. ○ Balb/c; ● C57B1/10Sn; △ DBA/1 and DBA/2

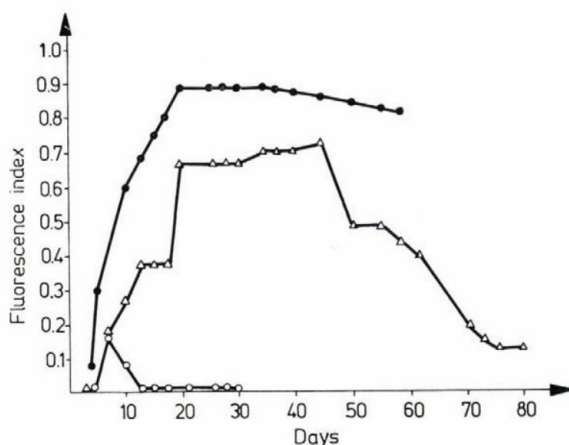


Fig. 2. Production of tumour-specific antibodies in mouse strains infected with Rauscher leukaemia virus. ○ Balb/c; ● C57B1/10Sn; △ DBA/1 and DBA/2

spleen = 1). The results are shown in Fig. 1. In Balb/c mice the disease ran a rapid course, the animals died within 40 days. DBA/1 and DBA/2 mice behaved similarly, showing a two-phase course: the initial progression was followed by a transient, partial remission then by an exacerbation. In the first phase about 10% of the mice died, whereas the exacerbation phase was fatal for all the remaining animals. In C57B1/10Sn mice a slight degree of transient splenomegaly was followed by a complete remission. With indirect membrane fluorescence (Fig. 2), the serum of Balb/c mice showed very low titres of antibodies 7 days after infection. In C57B1/10Sn mice the titre rose rapidly and remained at a high level. In DBA/1 and DBA/2 mice the tumour-specific antibody

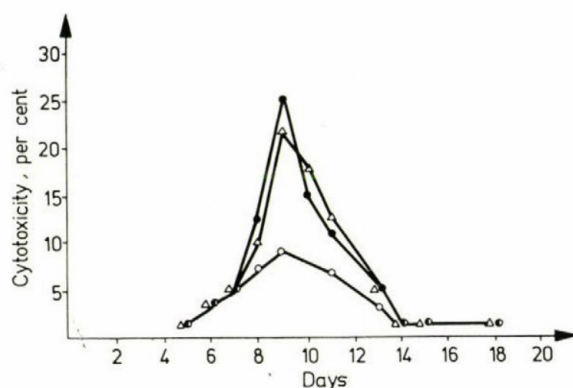


Fig. 3. Cellular immune response in mouse strains infected with Rauscher leukaemia virus.
 ○ Balb/c; ● C57B1/10Sn; △ DBA/1 and DBA/2

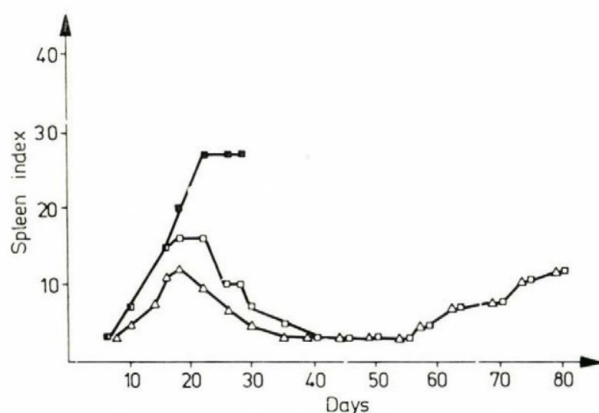


Fig. 4. Effect of anti-lymphocyte and anti-thymocyte serum on the pathogenesis of Rauscher leukaemia in DBA/1 and DBA/2 mice. △ control; □ anti-thymocyte serum; ■ anti-lymphocyte serum

response was also significant, but prior to the exacerbation the titre decreased gradually.

Lymphocytes prepared from the lymph nodes exerted cytotoxic activity between the 8th and 11th days after infection; the peak appeared on the 9th day (Fig. 3). C57B1/10Sn, DBA/1 and DBA/2 mice exhibited a similar degree of cellular immune response; the values in Balb/c mice were considerably lower than those in the above mouse strains.

Effect of anti-lymphocyte and anti-thymocyte serum on the course of infection and on tumour-specific immune response. The course of infection in Balb/c and C57B1/10Sn mice was not influenced by anti-lymphocyte and anti-thymocyte serum. In DBA/1 and DBA/2 mice both sera caused an increased progression of leukaemia (Fig. 4). While in animals treated with anti-thymocyte

serum only the initial splenomegaly increased, in those receiving anti-lymphocyte serum the course of the disease was considerably altered. DBA/1 and DBA/2 mice treated with anti-lymphocyte serum died in 30 days after the infection; post mortem there was a high degree of hepato-splenomegaly.

In C57B1/10Sn, DBA/1 and DBA/2 mice the production of tumour-specific antibodies was inhibited or delayed by anti-lymphocyte and anti-thymocyte serum (Figs 5 and 6). The effect of anti-lymphocyte serum was more marked than that of anti-thymocyte serum. In C57B1/10Sn mice given anti-thymocyte serum the antibodies appeared in significant titre at the end of the second week, whereas in mice injected with anti-lymphocyte serum only at the end of the fourth week. The difference was even more marked with DBA/1 and DBA/2 mice: in those treated with anti-thymocyte serum there was a considerable delay in antibody response; in the anti-lymphocyte serum group the tumour-specific antibodies were absent (Figs 5 and 6). Cellular immune response was blocked by anti-lymphocyte and anti-thymocyte serum in all mouse strains; lymphocyte suspensions prepared from the lymph nodes of serum-treated animals failed to exert cytotoxic activity.

Susceptibility of mouse strains to components of the Rauscher virus complex. The focus-forming effect of Rauscher virus was the most marked in Balb/c mice (100-fold as compared to that in DBA/1 and DBA/2 mice). In C57B1/10Sn mice the foci were absent. Accordingly, Balb/c mice were susceptible to both erythroblastosis (SFFV) and helper (LLV) viruses; DBA/1 and DBA/2 were susceptible to the erythroblastosis virus but not to the helper virus. The resistance of mouse strain C57B1/10Sn to the erythroblastosis virus is evident; its resistance to the helper virus may be assumed from the fact that in (Balb/c $\times$ C57B1/10Sn) F1 hybrids the spleen focus-forming capacity was identical with that in DBA/1 and DBA/2 mice. As (Balb/c $\times$ C57B1/10Sn) F1 hybrids are susceptible to the erythroblastosis virus, their 100-fold decreased focus-forming capacity may be attributed to a helper virus resistance allele originating from mouse strain C57B1/10Sn (Table I).

Table I

Spleen focus-forming capacity of Rauscher virus in different mouse strains

Mouse strain	FFU/ml	SFFV	LLV
Balb/c	$6.0 \pm 2.8 \times 10^4$	susceptible	susceptible
DBA/1, DBA/2	$2.5 \pm 0.7 \times 10^2$	susceptible	resistant
C57B1/10Sn	0	resistant	resistant
(Balb/c $\times$ C57B1/10Sn) F1	$2.7 \pm 1.4 \times 10^2$	susceptible	resistant

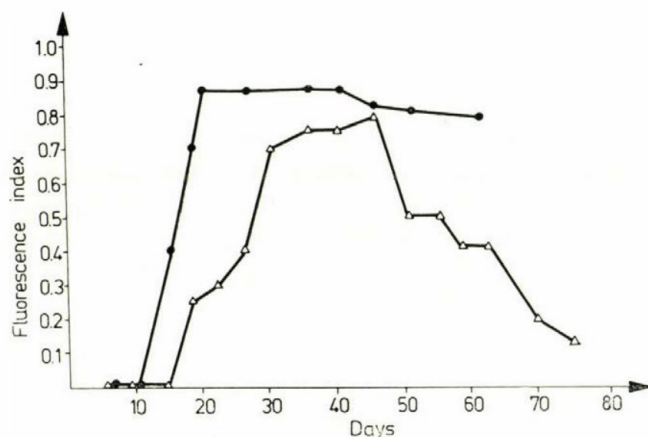


Fig. 5. Effect of anti-thymocyte serum on tumour-specific immune response in different mouse strains. $\triangle$ DBA/1 and DBA/2; $\bullet$ C57B1/10Sn

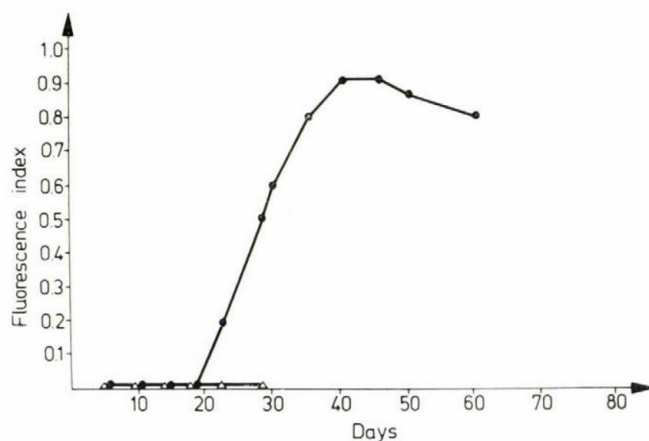


Fig. 6. Effect of anti-lymphocyte serum on tumour-specific immune response in different mouse strains. $\triangle$ DBA/1 and DBA/2; $\bullet$ C57B1/10Sn

Discussion

Repeated injection of anti-thymocyte serum exerts a toxic effect and decreases the development of splenomegaly in Rauscher leukaemic Balb/c mice [8]. The schedule applied in this study resulted in a selective immunosuppressive effect of anti-lymphocyte serum. Aspecific deaths were not observed in any of the mouse strains examined and in Balb/c mice not exhibiting a significant tumour-specific immune response the serum treatment failed to inhibit the progression of leukaemia.

In earlier studies [9] we have shown that Rauscher virus infection is followed by a prolonged appearance of leukaemic erythroblasts in C57B1/10Sn mice known as absolutely resistant to Rauscher leukaemia. As blocking the immune response in this mouse strain does not aggravate the course of the disease, it may be assumed that the resistance is determined at the level of the target cells and is due to a reversion of the tumour cells. In contrast, depending on the degree and duration of inhibition, elimination of the tumour-specific immune response in DBA/1 and DBA/2 mice is associated with a severe course of the initial progressive stage and absence of the remission. Accordingly, the comparative resistance of DBA mice appears to be due to the immune response. The cellular immune response is completely inhibited either by anti-lymphocyte or by anti-thymocyte serum; antibody production, however, is much more effectively blocked by anti-lymphocyte than by anti-thymocyte serum. The selective effect is probably associated with a difference between mouse T and B lymphocytes in surface marker proteins responsible for eliciting antibody production in the rabbit [10]. As the severity of the course of leukaemia is proportional to the inhibition of humoral immune response, in the elimination of the tumour cells the tumour-specific antibodies play a decisive role. Whether the antibodies act by inducing complement-dependent lysis or antibody-dependent cellular cytotoxicity [11], will be discussed in a forthcoming paper.

Studies on the genetic determination of susceptibility to mouse leukaemia viruses have shown a correlation between resistance to Tennant [12] Gross [13] and Friend [14] leukaemia viruses and H-2 genotype. It has been assumed that the Ir region of the H-2 complex, regulating the immunogenicity of tumour-specific antigens, is responsible for the effect. Accordingly, in different H-2 genotype mouse strains, the frequency of leukaemic symptoms and their remission depends on recognition of the antigens. However, DIETZ and RICH [16], using a large number of inbred mouse strains, failed to confirm the determining role of the H-2 locus in the regression of Friend leukaemia. GOTTLIEB *et al.* [17] have shown that the defective immunopoiesis in AKR mice carrying Gross leukaemia virus is not controlled by the H-2 gene complex. In an earlier study [3] we have assumed that remission due to immune response may depend on the resistance to the helper component of the Rauscher virus complex. Results presented in this paper have confirmed that assumption. Against erythroblastosis virus (SFFV) causing malignant transformation, Balb/c, DBA/1 and DBA/2 mice are uniformly sensitive, in other words in these mouse strains the malignancy of tumour cells is not eliminated spontaneously. However, depending upon susceptibility to the helper virus (LLV), the course of the disease varies markedly. In helper virus resistant DBA/1 and DBA/2 mice a significant tumour-specific immune response appears and causes a transient remission. In contrast, Balb/c mice susceptible also to the

helper virus, show a rapid progression of the disease in consequence of the absence of immune response. Since both Balb/c and DBA/2 mice are of genotype H-2^d, it is evident that the basis of the ability to produce antibodies is not determined by genotype H-2. From our studies [18] it would appear that genotype H-2 is not associated with the presence or absence, but with the duration of remission depending on the prolongation of the antigen expression. The mechanism by which susceptibility to the helper virus terminates the immune competence in mice sensitive to the erythroblastosis virus, awaits elucidation. Although strictly correlated with antibody production, the role of the thymus and T lymphocytes may be assumed, as in Rauscher leukaemic Balb/c mice IgG antibodies are absent whereas IgM antibodies are detectable in the early stage [19]. The observations of KOMITOWSKI *et al.* [20] that in NMRI mice, which are similar in susceptibility to Balb/c mice, after Rauscher virus infection there is a rapid decrease of T lymphocytes in the thymus, also support this hypothesis.

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GENERALIZED TRANSDUCTION OF SHIGELLA FLEXNERI BY CONVERTING PHAGE PE5

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Summary. Phage PE5, responsible for the conversion of type V antigen in *Shigella flexneri*, has the ability to produce generalized transduction. The correlation between phage multiplicity and the number of transductants, the specific inhibitory activity of anti-PE5 serum, and the lack of transduction in PE5 resistant recipients, indicate the role of phage PE5 in generalized transduction. Transduction of the R_{100-1} factor resulted in a non-transmissible tetracycline resistance segregation. The characteristics of the tetracycline resistance determinant suggest the possibility of integration.

Several phages, e.g. P22, ϵ^{15} , ϵ^{34} , can be characterized by the capacities for antigenic conversion and generalized transduction [1]. According to LEMINOR [2] every converting *Salmonella* phage is able to produce generalized transduction.

In a previous paper [3] some data were presented on the isolation, comparative morphology and biology of converting phages of *Shigella flexneri*. The lack of a convenient transducing phage in this particular subgroup justified a further study of the transduction caused by one of these phages.

Materials and methods

Bacteriophage. Phage PE5, a converting phage of type specific antigen "V" of *S. flexneri*, was used. Phage PE5 is non-UV-inducible and belongs to BRADLEY group A [4]. It was isolated by us [5].

Bacteria. *S. flexneri* var. Y, designed NTCC 4839 (Dr. G. GIAMMANCO) and its EMS induced mutants are listed in Table I. The nomenclature of generic markers follows the proposal of DEMEREC *et al.* [6]. A Hfr strain of *S. flexneri* 4b, selected by means of 4b-modified F factor designed UP4204, was also used [7].

Tetracycline resistant derivatives were selected from both the wild-type NTCC4839 and UP4204 strains by transduction of the R_{100-1} factor. This R factor was carried by strain D368 (Drs I. and F. ØRSKOV), an R_{100-1}^+ derivative of *Escherichia coli* K-12, and was transferred into NTCC4839 by conjugation. It is a derepressed (i^-) mutant, originally fi^+ [8] carrying sulphonamide, streptomycin, chloramphenicol and tetracycline (Sa, Sm, Cm, Tc) resistance.

Media. Difco Beef Extract in distilled water (0.3%), pH 7.2, termed as D-liquid, was optimal for the adsorption of phage PE5.

For cultivating strains and propagating phage, Lennox broth (LB) was used [9]: Tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; water, 1000 ml; pH 7.2. For solid medium, LB was completed with 1.5% Difco agar (LB agar). The top layer contained 0.5 to 0.6%, for replica plating technique, 0.8% agar.

For selection of auxotrophic markers a modified [7] Davis minimal medium was applied: glucose, 8 g; NaCl, 2 g; MgSO_4 , 0.05 g; $(\text{NH}_4)_2\text{SO}_4$, 1 g; KH_2PO_4 , 5.44 g; K_2HPO_4 , 16.49 g; water, 1000 ml. The analytically pure ingredients were dissolved to make a 10-times concentrated stock solution; its pH was set at 7.1 with KOH and sterilized by Seitz filtration. To the sterilized agar base the above-described salt solution was added together with the required amino acids and vitamins (20 $\mu\text{g}/\text{ml}$). The minimal medium was usually enriched with 0.25% LB.

For transfer or selection of the R factor, streptomycin, chloramphenicol, or tetracycline were added to LB-agar at a final concentration of 25 $\mu\text{g}/\text{ml}$.

Preparation of phage lysates. Lysates were prepared by the overlay method with 0.5 ml log phase bacterial culture (approximately 10^8 germs/ml) and 0.5 ml phage lysate (approximately 10^7 p.f.u./ml) in 4 ml medium (0.5% agar). The plates were incubated at 37 °C for 7 hr, then washed off with 3 ml of D-liquid, using glass rods. The bacteria were killed with chloroform and centrifuged at 10 000 g for 10 min. The lysates prepared in this way contained approximately $1\text{--}2 \times 10^{10}$ p.f.u./ml of phage. After a few days storage at 4 °C a slight decrease in p.f.u. titre could be observed.

Transduction. The log phase recipient culture was centrifuged, resuspended in D-liquid ($4\text{--}6 \times 10^8$ cells/ml), then lysate was added at m.o.i. 5–10. After incubation in water bath at 37 °C for 10 min the mixture was centrifuged at 10 000 g, the deposit was resuspended in minimal medium according to the initial number of recipient cells and dilutions were plated immediately on selective media. Results were evaluated after incubation at 37 °C for 48–72 hr.

The lysates were tested for sterility and their actual p.f.u./ml was determined. Frequency of transduction was calculated according to this actual p.f.u., not differing essentially from the output obtained by adsorbed phages. The wild-type NTCC4839 and most of its mutants showed an adsorption rate: $k = 1.8 \times 10^{-9}$ ml min⁻¹. The frequency of transduction was corrected by the eventual reversion frequency of the recipient strain.

Transductant-like colonies (with a minimum number of 100) were isolated, purified 2–3 times, and stability of the transduced marker, PE5 lysogeny, and antigenic conversion were tested. Lysogeny was determined on the basis of spontaneous phage liberation: transductants were replicated onto plates with a top-layer containing 0.8% agar and the indicator strain [10]. Antigenic conversion was tested by slide-agglutination in serum "V".

Production of antiphage serum. Antiphage serum was prepared by inoculating rabbits with purified and concentrated (CsCl gradient ultracentrifugation) lysate. Immunization was carried out according to ADAMS [11]. The antiphage serum was stored at -20 °C. Neutralization titre of the serum was: $k = 1038$ ml min⁻¹.

Interrupted mating experiments. Mating experiments were performed with the membrane-filter method [7].

"Deletion" by acridine-orange. Acridine-orange treatment was performed according to HIROTA [12]. Acridine-orange was diluted in LB at pH 7.6, to concentrations of 10, 20, 40, 80, 160 $\mu\text{g}/\text{ml}$. Each tube was inoculated with approximately 10^5 germs and after overnight incubation at 37 °C dilutions were plated, including an acridine-free control. After incubation, replicas were made on antibiotic-free and antibiotic-containing plates.

Results

1. Generalized transduction

Several markers were successfully transduced by phage PE5 at a frequency of 10^{-7} (Table II). The frequency of transduction varied with the use of different lysates or donor strains. In the latter case, the differences may have been due to a higher or lower rate of phage adsorption. E.g. the adsorption rate of strain NTCC4839 was: $k = 1.8 \times 10^{-9}$ ml min⁻¹, and that of the mutant UP163, $k = 4.9 \times 10^{-10}$ ml min⁻¹.

Analysis of the transductants proved that the transduced markers were stable. All transductants were lysogenic and antigenic convertant. It should be mentioned that 0.5–1.0 was the lowest m.o.i. applied in these experiments.

Table I*Phenotypic markers of strain S. flexneri* var. *Y* and its mutants

Wild-type NTCC4839	Nia ⁻
UP100	Nia ⁻ MetE ₁ ⁻
UP115	Nia ⁻ Met ₅ ⁻ Ser ₁ ⁻
UP132	Nia ⁻ Met ₅ ⁻ Gly ₁ ⁻
UP141	Nia ⁻ Met ₅ ⁻ Trp ₁ ⁻
UP149	Nia ⁻ Met ₅ ⁻ Arg ₂ ⁻ Ura ₁ ⁻
UP151	Nia ⁻ Met ₅ ⁻ His ₁ ⁻ Ade ₁ ⁻
UP153	Nia ⁻ Met ₅ ⁻ His ₁ ⁻ Cys ₁₄ ⁻
UP155	Nia ⁻ Met ₅ ⁻ Thr ₁ ⁻ St ₁ ^f
UP157	Nia ⁻ Met ₅ ⁻ Thr ₁ ⁻ Leu ₁ ⁻ Str ₃ ^r
UP159	Nia ⁻ Met ₅ ⁻ His ₁ ⁻ Ade ₁ ⁻ PE5 ^r
UP163	Nia ⁻ Met ₅ E ₁ ⁻ Ile ₁ ⁻ Str ₂ ^r

UP prefix means University of Pécs; PE5^r = resistance against phage PE5

Table II*Transduction of different markers by phage PE5*

Recipient strains	m.o.i.	Selected markers	Frequency of transduction per p.f.u.
UP155	6.0	Thr ⁺	3.9×10^{-7}
UP153	11.0	His ⁺	4.2×10^{-7}
UP153	11.0	Cys ⁺	1.2×10^{-7}
UP149	3.6	Ura ⁺	1.2×10^{-7}
UP132	5.0	Gly ⁺	1.7×10^{-7}
UP115	7.9	Ser ⁺	4.1×10^{-7}
UP141	4.6	Trp ⁺	2.8×10^{-7}
UP157	6.1	Leu ⁺	1.6×10^{-7}
UP163	4.6	MetE ⁺	6.1×10^{-8}
UP163	4.6	Ile ⁺	3.3×10^{-7}

2. Factors influencing or inhibiting transduction

Although the transduction observed was not unexpected, it was necessary to prove the role in it of PE5.

At first, a correlation could be demonstrated between the applied multiplicity of the phage (m.o.i.) and the resulting number of transductants (Fig. 1). The increase in number of transductants was almost linear with the

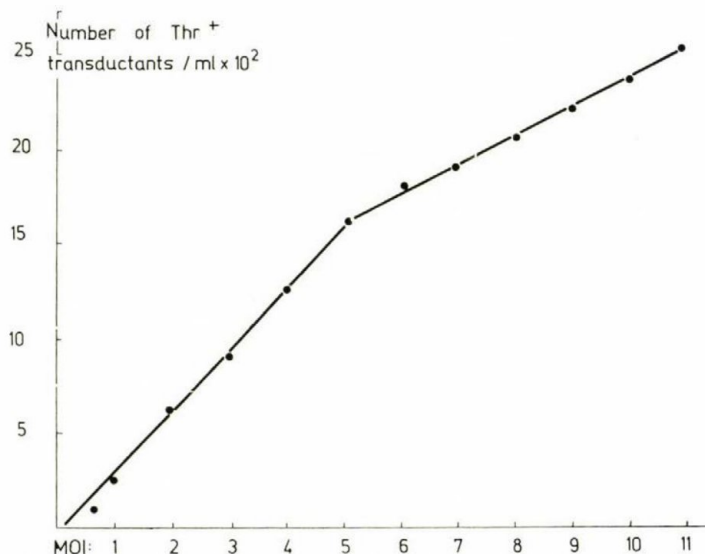


Fig. 1. Effect of the multiplicity of phage PE5 on the number of Thr⁺ transductants. Recipient: UP155 (3.4×10^8 germs/ml)

m.o.i. up to a value of 5–6. Beyond this a slow plateau formation was observed. This characteristic curve may reflect the gradually increasing killing effect of phage PE5.

Treatment of host bacteria with a specific antiphage serum before phage adsorption prevented, and after phage adsorption did not influence, the occurrence of transduction. Normal serum had no effect on transduction (Table III).

Table III

Effect of antiphage serum on transduction by phage PE5

Treatment	Surviving ratio of phage PE5, per cent	Frequency of transduction/p.f.u.	
		His ⁺	Cys ⁺
Antiphage serum before adsorption	0.001	$< 2.0 \times 10^{-10}$	$< 2.0 \times 10^{-10}$
Antiphage serum after adsorption	—	9.8×10^{-7}	3.3×10^{-7}
Normal rabbit serum before immunization	100	8.0×10^{-7}	2.7×10^{-7}
Without antiphage serum	100	7.1×10^{-7}	2.9×10^{-7}
Reversion control	—	1.3×10^{-8}	$< 1.0 \times 10^{-9}$

The value of antiphage serum PE5 was $k = 1038 \text{ ml min}^{-1}$, used in a dilution of 1 : 10; m.o.i. = 7.2. The rate of reversion was calculated on the basis of the number of recipient cells. The number of transductants is corrected by the number of revertants observed.

In a further control experiment the lysate had been propagated on the recipient strain before transduction was attempted. A total loss of transduction capacity was observed. In the case of a mutant strain (UP159), resistant to PE5 phage, no transduction occurred.

3. Co-transduction with phage PE5

The possibility to investigate co-transduction of chromosome markers in *S. flexneri* is limited, the map positions of the markers being practically unknown. Two marker-pairs, Thr-Leu and MetE-Ile were, however, suitable for such investigations. According to our conjugation experiments with Hfr strain UP4204, these markers are closely linked on the chromosome of *S. flexneri*. Linkage between Thr and Leu markers was about 78% and the MetE and Ile markers linked in 52%, while co-transduction was not observed (< 0.05 and $< 0.02\%$, respectively).

Due to the difficulty in co-transduction of chromosome markers we had to investigate the transduction of an R factor. By means of conjugation, the R_{100-1} factor was transferred into the wild-type strain NTCC4839. In view of the sulphonamide resistance, the observations had to be focussed onto the chloramphenicol and tetracycline markers. Using this derivative as R^+ donor, transduction was performed by phage PE5. The transfer of tetracycline resistance was found at a frequency of 5.2×10^{-9} p.f.u./ml; no Cm^r or $Cm^r \cdot Tc^r$ transductants were observed ($< 2.6 \times 10^{-10}$).

The stability of Tc^r marker and lysogeny by phage PE5 was determined on 15 purified clones. Lysates of these clones were prepared as follows: 7 hr LB-cultures of the clones were killed with chloroform, centrifuged, 0.25 ml aliquots of the supernatants (lysates) were mixed with the recipient strain and plated. The majority of the clones showed a high number of transductants. In one case (NTCC4839 Tc^r/l) the frequency of transduction was as high as 1.7×10^{-2} p.f.u./ml. In the case of five Tc^r clones the transmissibility of the determinant was examined by conjugation, using the phage resistant mutant UP159 as recipient. Tc resistance could not be transferred in this manner.

On the basis of our working hypothesis concerning the integration of Tc^r determinant with the prophage, transduction of the R factor was performed by using the Hfr strain UP4204 as recipient. Analysis of 15 Tc^r clones of UP4204 proved the expected marker stability and lysogeny. One of them, UP4204 Tc^r/l showed neither spontaneous loss of resistance ($< 0.09\%$) nor a loss caused by acridine-orange ($< 0.08\%$).

Interrupted mating experiments were performed by using Hfr UP4204 Tc^r/l , and as a control NTCC4839 Tc^r/l donor strains, and the UP159 phage resistant mutant as recipient. Selection was made for the Tc^r character (Table IV). Transduction and reversion were controlled simultaneously. As expected, no conjugational transfer was observed by the NTCC4839 Tc^r derivative, even

Table IV
*Interrupted mating experiments between Tc^r donor strains and
 UP159 (PE5^r) recipient strain*

Mating time, min	Frequency of recombination/initial donor cells	
	Hfr UP4204 Tc ^r /I	NTCC4839 Tc ^r /I
20	$< 7.1 \times 10^{-8}$	$< 7.1 \times 10^{-8}$
30	$< 7.1 \times 10^{-8}$	$< 7.1 \times 10^{-8}$
40	4.2×10^{-6}	$< 7.1 \times 10^{-8}$
60	9.8×10^{-5}	$< 7.1 \times 10^{-8}$
120	3.1×10^{-4}	$< 7.1 \times 10^{-8}$
Reversion control	$< 1.0 \times 10^{-8}$	$< 1.0 \times 10^{-8}$
Transduction control	$< 1.0 \times 10^{-9}$	$< 1.0 \times 10^{-9}$

Reversion control was calculated by the number of the recipient cells. Transduction control, using the phage resistant recipient strain, is expressed per p.f.u.

after two hours mating time. On the other hand, Hfr strain UP4204 transferred the Tc^r determinant, with a definite entry time (between 30 and 40 min), and with a characteristic rise in the number of exconjugants. Of a sample taken after 60 min mating, 100 purified exconjugants were analysed. The result showed the stability of Tc^r marker, lysogeny and antigenic conversion in all of the clones.

Discussion

The data presented prove that generalized transduction produced by phage PE5 was in correlation with phage multiplicity and could be prevented by specific antiphage serum, by propagation on the recipient, and by using phage resistant recipient cells.

Abortive transduction, predominant in transductions produced by several phages, as e.g. by P1 [13] or P22 [14], could not be observed. This negative finding may have had technical reasons in that the heavy "background" of the plates necessary for the cultivation of shigellae may conceal such transductants.

The lack of co-transduction in the case of chromosome markers means that phage PE5 does not have a higher capacity of co-transduction than e.g. phage P22 [15]. Because of the limited possibilities of investigating other, more closely linked, markers, experiments were carried out with the R₁₀₀₋₁ factor.

These experiments indicated the separate transfer of Tc^r determinant, a lack of transmissibility by conjugation, successful transfer with an Hfr

strain with fixed entry time and acridine-dye resistance. Some further observations, like the lysogeny of Tc^r transductants and the high capacity of transduction of their lysates, suggest the hypothesis that integration proceeds together with the prophage (at the *att* site), so that the observed high frequency of transfer could be caused by a specialized transduction.

According to the mating experiments, the entry time of Tc^r marker is between 30 and 40 min. Localization of *S. flexneri* type antigens [16] with the probable identity with the *att* sites [17] point to the *pro-purE-lac* region. The entry time of this segment in the case of our Hf^r strain is expected between 30 and 35 min, in good agreement with the data obtained.

R factors can be transduced by phage P1 [18, 19]. Segregation of R factors is infrequent and the transductants carry a transmissible plasmid [20]. On the other hand when, at least in the case of fi^+ , the R factors are transduced by phage P22, segregation of Tc^r determinant and the loss of transmissibility are often recorded [21]. According to WATANABE [20] in these transductants not only the transfer factor is often missing but also a determinant of the autonomous replication. Replication therefore occurs only after integration at the site of the *proB-att* P22 region. By constructing a linkage map of R_{100-1} factor [22] it was shown that one of the replication determinants (*repA*) was localized far from the Tc^r determinant. A similar situation exists in the case of epsilon phages. HARADA *et al.* [23] observed segregation of the Tc^r determinant in transductants, with a resistance to acridines and a lack of transmissibility.

By phage P22, specialized transduction was achieved with markers near to the *att* site [14, 24]. Among these markers was the Tc^r determinant of R22 factor, too [25, 26]. The mechanism of the phenomenon was elucidated by TYE *et al.* [27, 28].

To prove the transduction of R factor with phage PE5, further studies are needed, which may have a special significance in showing the localization of the prophage.

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EFFECT OF THE INTERFERON INDUCER TILORONE IN INBRED CBA MICE

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Summary. Tilorone hydrochloride (200 mg/kg) was administered intragastrically to inbred CBA mice. After 5, 18 and 48 hr the number of circulating leucocytes and peritoneal cells as well as the migration of unstimulated peritoneal cells, the blood corticosteroid level and interferon production were investigated. In spite of the considerable decrease of the number of mononuclear cells in the blood and of polynuclear ones in the peritoneal exudate, the drug induced production of circulating interferon and stimulated its synthesis by peritoneal cells. The blood corticosteroid level and the mast cell count in the peritoneal cavity were significantly elevated, but the migration of peritoneal cells in antigen-free medium decreased.

Tilorone hydrochloride was described by KRUEGER and MAYER [1, 2] as an orally active interferon inducer. Tilorone protected mice against infection with DNA and RNA viruses [3–5] and rats against Walker carcinoma [6]. It was also shown that tilorone elevated 19S and 7S antibody production to sheep red blood cells [7, 8] and to *Escherichia coli* endotoxin [8], and inhibited the tuberculin skin reactions and adjuvant-induced arthritis [8]. A selective depletion of thymus dependent areas was also reported [9].

A study has been made of the effect of tilorone on the number of circulating leucocytes and peritoneal cells, as well as on the migration of unstimulated peritoneal cells, blood corticosteroid levels and interferon production.

Materials and methods

Administration of tilorone. Tilorone hydrochloride (Merrell National Lab., Cincinnati, Ohio, U.S.A.) was dissolved in water and administered intragastrically in 200 mg/kg doses to inbred CBA mice weighing 20–25 g.

Interferon assay. Sera for interferon assay were obtained from the retro-orbital venous plexus. Sera of groups consisting of 5 mice were pooled.

Peritoneal exudate cells were obtained, washed 2 times and suspensions containing 2×10^6 cells/ml were prepared in Parker's TC 199 medium. After freezing and thawing 3 times the cell-free supernatant was used for interferon assay.

Interferon levels were determined by reduction of the cytopathic effect of Semliki Forest Virus in mouse L-cells as described previously [3, 5].

Counting of cells. (a) *Peripheral leucocytes.* Blood was obtained from the tail vein and the leucocytes were counted in a Buerker chamber. Of each animal, 200 leucocytes stained according to Giemsa were analysed.

(b) *Mast cells.* Three ml of washing medium (0.05 M phosphate buffer pH 7.0, 0.38% sodium citrate in 0.58% NaCl containing 1 mg of phenol red per 100 ml) was injected into the

peritoneal cavity of mice. Cells were withdrawn and diluted to 1 : 5 with the same washing medium supplemented with 10% calf serum. Five μ l of the cell suspension in thick drops were stained with Alcian blue-saffranin [10].

(c) *Peritoneal cell number* was counted 7 days after sterile liquid paraffin injection as well as without any stimulation. Cells withdrawn in 6 ml Hanks' solution (pH 8) were stained with Giemsa.

Macrophage migration. Three ml of sterile liquid paraffin were injected into the peritoneal cavity of 22-week-old male mice. After 7 days tilorone (200 mg/kg) was administered intragastrically and 5, 18 and 48 hr later the mice were exsanguinated by decapitation. Exudate cells were withdrawn from the peritoneal cavity after injection of 6 ml Hanks' solution (pH 8) supplemented with 5 units/ml of preservative-free heparin (Richter, Budapest). The cells were centrifuged at 250 g at 4 °C for 10 min and liquid paraffin was removed with Pasteur pipettes. The cell pellets were washed 2 times by centrifugation in Hanks' solution and final resuspension was made in Parker's TC 199 medium (pH 7.4) supplemented with 20% calf serum. Peritoneal cells of 6 mice were pooled, counted and made up to a concentration of 3×10^6 cells per capillary (Portex surgical quality polythene capillary tubing, PP 160, diameter 1.2 mm). The capillaries were put into polythene culture chambers which were covered with two round borosilicate cover glasses (10 mm in diameter, thickness 0.2 mm) and the chambers were filled with 0.25 ml Parker's TC 199 medium supplemented with 20% calf serum. The chambers were kept at 37 °C, then 18 hr later the areas of migration were magnified 1 : 20 by a projector on standard paper, and the paper pieces were cut out and weighed.

Determination of the blood corticosteroid level was made according to MATTINGLY [11].

Results and discussion

Figure 1 demonstrates the effect of tilorone on the peripheral leucocyte count. The total number of leucocytes and that of polynuclear and mononuclear cells is given separately in per cent of the control values. Every point represents the average of 10–15 mice. A sharp decrease to 58% in the total number of leucocytes was observed after 5 hr of tilorone administration with a subsequent reduction to 30–32% at the 18th and 48th hr.

The decrease in the leucocyte count on the first day of investigation was mainly due to the reduction of mononuclears. The number of polynuclears

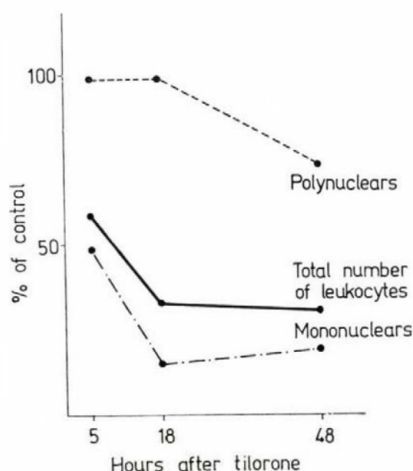


Fig. 1. Effect of a single oral dose of tilorone (200 mg/kg) on circulating leucocytes in CBA mice

did not change at that time, and a decrease of the polynuclear count could only be observed after 48 hr.

Figure 2 shows the effect of tilorone on the number of peritoneal cells. The decrease of the number of peritoneal exudate cells started later than that of the blood leucocytes. After 18 hr a decrease of the total number of cells, due to the decrease of both the mononuclear and polynuclear counts, could be seen; the reduction in the number of polynuclear cells was more marked than that of the mononuclears.

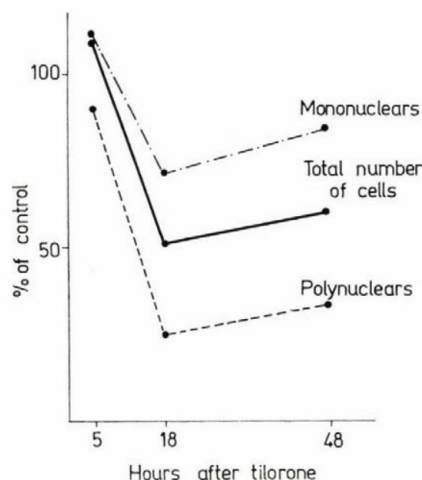


Fig. 2. Effect of a single oral dose of tilorone (200 mg/kg) on the number of paraffin-induced peritoneal cells in CBA mice

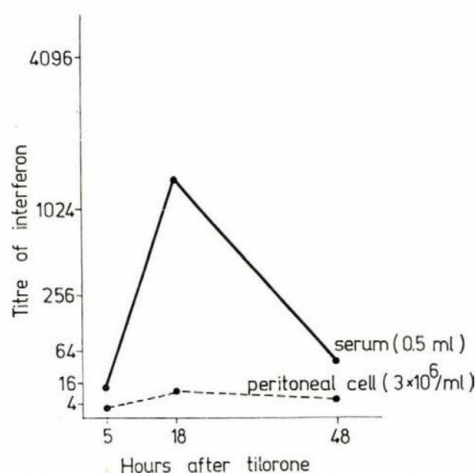


Fig. 3. Interferon levels in serum and in peritoneal cells of CBA mice after a single oral dose of tilorone (200 mg/kg)

Table I

Migration of unstimulated peritoneal cells and plasma corticosteroid contents of control and tilorone-treated mice

Treatment	Time after treatment, hr	Migration of peritoneal cells*		Corticosteroid content in plasma* $\mu\text{g}/100\text{ ml}$
		migration areas, mg	migration inhibition, per cent	
—	—	265		27**
Tilorone	5	159	40	41.8
—	—	230		26
Tilorone	18	138	40	59.5
—	—	280		15.6
Tilorone	24	140	50	17.8

* Peritoneal cells as well as plasmas of 6 mice were pooled for investigation

** Normal corticosteroid level in mouse plasma is 9.7–30.0 $\mu\text{g}/100\text{ ml}$ [12]

Interferon induction by tilorone is presented in Fig. 3. It can be seen that in spite of the considerable decrease of the number of circulating mononuclear and polynuclear cells in the peritoneal exudate, tilorone stimulated the production of circulating interferon and the interferon synthesis by peritoneal cells. Maximum interferon levels in serum and peritoneal cells were detected 18 hr after tilorone administration.

The migration areas of peritoneal cells of tilorone-treated mice proved to be 40 to 50% smaller than those of the control animals (Table I). In contrast to the transient effect of tilorone on interferon production by peritoneal cells, its inhibitory effect on cell migration, lasting at least for 48 hr, was more stable.

After tilorone administration, abnormal mononuclear cells with granules, stained by Giemsa and showing affinity for Sudan black, were found in the blood. These cells were positive for acid phosphatase and acid mucopolysaccharides and displayed vacuolation [13, 14]. According to DE DUVE and WATTIAUX [15] acid phosphatase-positive particles are likely to be lysosomes, which might be stabilized by cortisone to prevent the release of enzymes [16]. These data allow to suppose that a certain activation of lysosomal function may have occurred in the circulating leucocytes after tilorone treatment and prompted us to check the number of mast cells in the peritoneal exudate and to determine the blood corticosteroid level.

Figure 4 shows the effect of tilorone administration on the peritoneal mast cell count. In the three periods investigated, three families of mice were used and this explains the variations in the control values. But the figure clearly

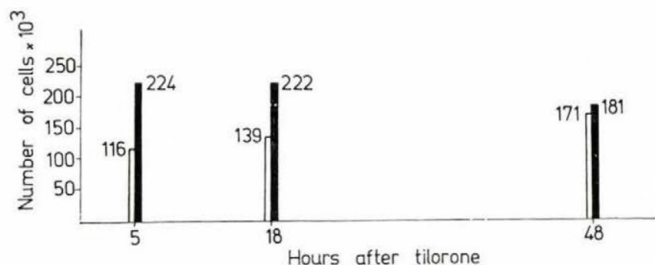


Fig. 4. Effect of tilorone on the number of mast cells in peritoneal exudate of CBA mice. Open columns: control mice; solid columns: tilorone-treated mice

shows the marked increase of the mast cell count 5 and 18 hr after tilorone administration.

The increase in the mast cell count was due to an increase of both mature and young forms. After 48 hr, ruptured mast cells could also be observed.

Table I also shows elevated blood corticosteroid levels during the first day of tilorone administration. This increase could be observed as early as 5 hr after tilorone administration, then after a transient elevation to above 100% in the 18th hr the corticosteroid level returned to the control value.

The presented results support and supplement the observation of other authors concerning the broad activity spectrum of tilorone. We could show that a single dose induced in mice a certain redistribution of circulating and peritoneal leucocytes, a transient increase in the blood corticosteroid level and in the number of mast cells in the peritoneal cavity as well as a decrease of the migration of unstimulated peritoneal cells.

The increase in the blood corticosteroid level induced by tilorone seems to antagonize the activation of lysosomal function [15]. The transient increase in the number of mast cells and the decrease in the migration of peritoneal cells may indicate changes of the cell mediated immune reactions.

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PRECIPITATING ANTIBODIES TO CRIMEAN HAEMORRHAGIC FEVER VIRUS IN HUMAN SERA COLLECTED IN HUNGARY

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Summary. Agar-gel diffusion precipitation test was carried out with Crimean Haemorrhagic Fever (CHF) virus as antigen in serum samples collected in four areas of Hungary from 587 persons working in contact with animals, and in 169 animal sera collected in Hajdú-Bihar county, Hungary. Antibody was found in 17 human sera. All these but one, originating from a slaughterhouse worker in Budapest, had been collected from Hajdú-Bihar county. The highest titre was 1 : 16. All the animal sera proved to be negative. It is suggested that the CHF virus or a closely related virus is present, and may cause human infections in Hungary.

Crimean Haemorrhagic Fever (CHF) has been defined by CHUMAKOV [1] as an entity of viral aetiology in connection with a Crimean epidemic. In Hungary, we found antibodies to the CHF virus in serum samples collected from cattle and sheep [2]. In the present work, we tested serum samples from humans who, owing to their occupation, were thought to have been exposed to CHF virus.

Materials and methods

From the Hungarian counties Heves, Veszprém and Hajdú-Bihar and from the Budapest Slaughterhouse, 587 human serum samples were collected in 1972–1975. In addition, 161 bovine sera and 8 horse sera were collected from Hajdú-Bihar county. The donors living in the country were employed in agriculture, forestry and animal farms. The serum samples were kept at 4 °C until used within 1–2 months (sera from Hajdú-Bihar county and Budapest) or in the frozen state until used at a later time (sera from Veszprém county and Heves county).

Antigen. The Piatnitskaya strain of CHF virus was cultivated in vervet monkey kidney cell cultures and concentrated 300-fold [3] by polyethylene-glycol precipitation. The strain had been kindly supplied by the Institute for Poliomyelitis and Viral Encephalitis Research of the Academy of Medical Sciences of the U.S.S.R., Moscow.

Agar-gel diffusion precipitation (AGDP) test. OUCHTERLONY's original method was used [4]. A twofold dilution series of each sample was tested. A borate solution was used as diluent.

Results

CHF antibodies were demonstrated in 17 human sera. Of these 16 had been collected in Hajdú-Bihar county and one in the Budapest Slaughterhouse (Table I). Twelve of the seropositive donors were animal attendants, three were veterinarians and one was a gardener.

Table I

Distribution by domicile and occupation of the subjects tested for antibodies to CHF virus

Occupation	Area of domicile				Total
	Heves county	Veszprém county	Hajdu-Bihar county	Budapest	
Animal attendant	—	—	12/276*	—	12/276
Veterinarian	—	—	3/6	—	3/6
Slaughterhouse worker	—	—	—	1/109	1/109
Gardener	—	—	1/4	—	1/4
Forest worker	0/156	0/24	—	—	0/180
Hunter	—	0/12	—	—	0/12
Total	0/156	0/36	16/286	1/109	17/587

* No. of seropositives/No. of persons tested

Table II shows the distribution by age and county. The donors of the blood samples were 15 to 71 years of age. No specific age-distribution could be demonstrated.

Table II

Distribution by domicile and age of persons examined, and those positive for CHF precipitating antibodies

Age, year	Area of domicile				Total
	Heves county	Veszprém county	Hajdu-Bihar county	Budapest	
15-20	0/14*	0/1	1/11	0/8	1/34
21-30	0/37	0/8	4/58	1/32	5/135
31-40	0/63	0/7	4/75	0/17	4/162
41-50	0/33	0/12	2/92	0/23	2/160
51-60	0/7	0/7	2/39	0/29	2/82
61-70	0/2	0/1	2/10	0/0	2/13
71	0/0	0/0	1/1	0/0	1/1
Total	0/156	0/36	16/286	1/109	17/587

* No. of seropositives/No. of persons tested

The antibody titres of the positive sera ranged from 1:1 to 1:16 (Table III).

Some sera formed a single precipitation line, others formed an additional one (Fig. 1). None of the animal sera proved to be positive.

Table III
CHF antibody titres of the positive human sera

Antibody titre*	1	2	4	8	16
No. of sera with the indicated titre	7	3	2	1	4

* Reciprocals

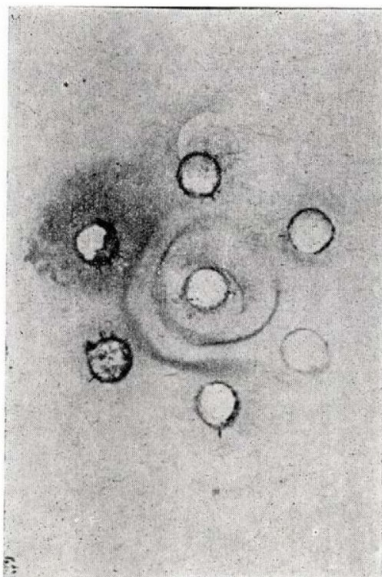


Fig. 1. Precipitation lines with positive sera

Discussion

In the Soviet Union and in Bulgaria, the vectors of CHF virus are ticks, viz., *Hyalomma* and *Rhipicephalus* species, *Dermacentor marginatus*, *Ixodes ricinus*, *Boophilus calcaratus*, and *Haemophysalis punctata* [5, 6]. In Hungary, among the prevailing ticks, *Dermacentor marginatus*, *Dermacentor reticulatus*, *Ixodes ricinus*, *Ixodes trianguliceps* and *Haemophysalis concinna* may come into account as vectors [7]. In ticks the virus may persist for 13 months or more and transovarian transmission is possible [8]. The vector may transmit the virus from the source of infection by blood sucking to both wild [9] and domestic animals (horse, cattle, sheep, donkey and goat).

The infection, though symptomless in domestic animals, induces serological responses in them [10]. Attempts to demonstrate carriership in domestic animals failed in the Astrakhan area of the Soviet Union, whereas the virus could be isolated from cattle and goat sera in Nigeria and Kenya [11].

The virus is transmitted to man by vectors or by direct contact with the blood of patients in the viraemic stage of the disease [12]. Laboratory workers have been infected even intranasally [5, 13].

CHUMAKOV *et al.* in 1974 [cit. 14] were the first to publish a human case in which the infection occurred in connection with cattle slaughtering. In man, 15% of the infections were found to be latent while the majority of the cases showed the haemorrhagic syndrome.

In the present study, eight of the seropositive persons had been in contact with ticks, the skin of slaughtered animals and/or had stayed in neighbouring forests a few months before the blood sample was taken. Some of them mentioned fever or epistaxis without fever. It remains open whether these symptoms of the supposed CHF or coinciding symptoms are due to other factors.

In response to CHF infection, virus-neutralizing, precipitating and complement-fixing (CF) antibodies are formed. Virus-neutralizing antibodies persist in serum longer than precipitating antibodies. Since, however, the AGDP test is time-saving and easy to perform, it is preferable to the neutralization test in screening for CHF antibodies. The CF test is not suitable for this purpose because CF antibodies have been found to disappear by the 24th day after infection [6, 10, 11, 15].

In 1974, we succeeded in demonstrating antibodies to CHF virus in serum samples from a cattle and 15 sheep in Hajdú-Bihar county [16], and 16 of 17 positive human blood samples in the present study originated from the same county. Although none of the animal sera tested in the present study proved to be positive, a CHF focus is supposed to be present in that county.

One of the seropositive subjects was a slaughterhouse worker. In the literature one CHF has been reported from a similar institution (Chimkent, Kazakh Soviet Socialist Republic [17]). The patient had skinned slaughtered animals. However, contact with ticks outside the establishment could be excluded neither in that case nor in ours.

The data available are unsatisfactory to reveal the sources of CHF infection in Hungary. The presence of antibodies in both humans and animals nevertheless suggests that the CHF virus, or an antigenically closely related virus, is present and may infect humans in this country.

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ANTIGENIC CHANGES IN *PSEUDOMONAS AERUGINOSA* IN VIVO AND AFTER LYSOGENIZATION IN VITRO*

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Summary. *Pseudomonas aeruginosa* cultures not conforming in antigenic structure to established serogroups were isolated from infants and from 23 O antigen type strains lysogenized with 22 different phages *in vitro*. The derivatives, characterized by smooth colonial form and heat stability, retained at least part of the antigens of the parent strains and became agglutinable in certain heterologous sera. All derivatives showed immunoelectrophoretic patterns identical with those of the parent strains. Antigenic analysis showed the presence of factors in the derivatives identical with (italicized figures), bilaterally related to (non-bracketed figures) or unilaterally related to (bracketed figures) O antigens of LÁNYI's schema: (i) changes *in vivo*, O1 → O1, (O8); O11a,11b → O11a,11c; (ii) changes *in vitro*, O1 → O1, (O8); O4a, 4b → O4a,4b,O1,O8,(O10a),O11; O4a,4b → O4a,4b,O1,O8,O10a,O11; O4a,4c → O4a,4c, O1,(O8),(O10a),(O11). The antigenic changes were accompanied usually by a loss of sensitivity to several typing phages and the lytic spectrum of phages released from the derivatives had become narrower. The findings suggest that multiple agglutinability of *P. aeruginosa* frequently encountered in nature is associated with phage action.

Bacteriologists using the serological typing of *Pseudomonas aeruginosa* frequently meet strains not corresponding in antigenic structure to established serogroups. In the history of pseudomonas serology the problem of reproducibility arose again and again, and today, when typing is made on the basis of well-elaborated antigenic schemata, it still presents difficulties.

In hospital units where *P. aeruginosa* is predominant, usually more than one different serogroups of the organism are encountered. In the course of prolonged treatment, especially in chronic respiratory wards, many or all of the nosocomial serogroups may colonize the same patient. The appearance of one or more new serogroups is sometimes erroneously interpreted as a change in antigenic structure. Alteration of one defined serogroup to another defined serogroup in patients has not been proved. Changes occurring frequently are those characterized by the appearance of multiple agglutinability, when the culture retains its smooth colonial form, but becomes agglutinable in one or more group sera not reacting with the parent strain. Antigenic changes have been shown by several authors and on the basis of the source of the

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variants they can be classified into three groups: (i) changes occurring in patients, animals and in their environment; (ii) changes induced by bacteriophages *in vitro*; (iii) changes due to cultivation on artificial media.

Materials and methods

Bacterial strains. *P. aeruginosa* strains showing unusual antigenic structure were isolated in the course of routine epidemiological tracing of infections occurring in infants' wards. For lysogenization experiments, 23 *P. aeruginosa* cultures (No. 170001-170023) representing LÁNYI's [1, 2] O antigens were used.

Bacteriophages. Twenty *P. aeruginosa* phages (No. 7, 16, 21, 24, 31, 44, 68, 73, F7, F8, F10, 109, 119x, 352, 1214, M4, M6, C11, C18, C21) were received from the Central Public Health Laboratory, Colindale, London. Seventeen of them originated from the collection of Col. R. B. LINDBERG, U.S. Army Surgical Research Unit, Fort Sam Houston, U.S.A.; 1 was isolated in Greece and 2 at Colindale. Phages 2 and C188 and their propagating strains were supplied by Dr. L. SJÖBERG, National Bacteriological Laboratory, Stockholm. The phages were propagated by the use of the soft agar method and their lytic spectra were checked on the test strains.

Culture media. Soft agar: beef extract (Central Slaughterhouse, Budapest), 7.5 g; Witte peptone, 10 g; agar, 12 g; the pH was adjusted to 7.2 with 0.1 M Tris buffer. For serum absorption experiments and for the preparation of heated suspensions the cultures were grown on nutrient agar, and for slide agglutination on blood agar [3].

Lysogenization. Soft agar plates were flooded with 3 hr broth cultures. After drying, the plates were spotted with concentrated phages. Colonies growing within the plaques after 18 hr incubation were subjected to 3 subsequent streakings and single colony isolations.

Induction of prophage. A thin layer of bacterial suspension (5 ml broth culture in a Petri dish 90 mm in diameter) was irradiated with the Medicor BLF12 ultraviolet lamp (intensity, 0.2 A; distance from the liquid surface, 300 mm; duration of irradiation, 60 s). The irradiated broth was incubated at 37 °C for 4 hr, then centrifuged. The supernatant was diluted 10^{-1} and dropped onto plates inoculated with the propagating strains. The lytic spectra were compared with those of the lysogenizing phage. Prophage induction was performed also by irradiating saline suspensions (3 ml, OD = 0.2) with the same method; to 2 ml amounts of the irradiated and control suspension 2 ml aliquots of double-strength broth were added and after incubation at 37 °C for 4 hr the supernatants were plated at 10^{-1} , 10^{-2} and 10^{-3} dilution as described above.

Phage typing of the isolates was performed at routine test dilution.

Serological analysis. Screening of the isolates was performed by slide agglutination test in O sera according to LÁNYI's antigenic schema [1]. Representatives of cultures showing serological reactions different from that of the parent strain were analysed for O antigens by tube agglutination using heated (100 °C for 2½ hr + 130 °C for 1 hr) suspensions and by cross-absorption test [1, 2].

Immunoelectrophoretic grouping was carried out by the method of LÁNYI *et al.* [3].

Freeze-drying of the parent strains and their derivatives was performed in the Edwards Model 30 PI centrifugal freeze-drier. Each ampoule contained approximately 10^9 organisms in 0.1 ml volume. The suspending fluid contained 1 volume of rabbit serum (Seitz-filtered and inactivated at 56 °C for 2 hr) and 1 volume of "CI" solution (cysteine HCl, 1 g; ascorbic acid, 1 g; inositol, 30 g; K_2HPO_4 , 3.5 g; distilled water, 1000 ml; pH 7.0; 110 °C 20 min). This mixture has been used for 15 years in the Hungarian National Collection of Medical Bacteria and found excellent for the preservation of a wide variety of microorganisms.

Results

1. Antigenic changes of *P. aeruginosa* in patients

In an infants' ward (Municipal Hospital, Debrecen) surveyed for the spread of *P. aeruginosa*, in addition to the predominating serogroup O1 strains, a number of isolates were encountered which agglutinated not only in serum

O1, but also in serum O8. Antigenic analysis of representatives of the probable parent strain and the variants is presented in Table I. It is evident that the parent and the variant strains have closely similar serological features: they are identical in antigen O1 and in the relationship to antigen O8 the difference is more quantitative than qualitative; in this respect it is remarkable that the homologous agglutinins from serum O8 were entirely removed by isolate Lak 744, whereas the parent strain only decreased its titre considerably.

Table I

Antigenic structure of P. aeruginosa isolate Lak 744
Probable parent strain, *P. aeruginosa* O1

Strain	Serum							
	O1		O8			Lak 744		
	unabs.	abs. by Lak 744	unabs.	abs. by O1	abs. by Lak 744	unabs.	abs. by O1	abs. by O8
O1	2560	0	40	0	0	5120	0	1280
O8	40	0	1280	160	0	80	0	0
Lak 744	2560	0	160	0	0	5120	0	1280

O antigens of strain Lak 744: identical with O1; unilaterally related to O8

In another infants' ward (Árpád Hospital, Budapest) where *P. aeruginosa* serogroups O3a,3d,3f, O4a,4b, O4a,4d and O11 predominated, part of O11 strains reacted also in serum O8. Antigenic analysis (Table II) confirmed a unilateral partial connection to O8 and revealed an a, b-a, c-type relationship between the isolate and the probable parent strain: in addition to antigen O11a shared by both cultures, each contained a distinct factor absent from the other.

Table II

Antigenic structure of P. aeruginosa isolate Ar 122
Probable parent strain, *P. aeruginosa* O11a,11b

Strain	Serum						
	O11		Ar 122		O8		
	unabs.	abs. by Ar 122	unabs.	abs. by O11a,11b	unabs.	abs. by O11a,11b	abs. by Ar 122
O11a,11b	1280	640	1280	0	0	0	0
Ar 122	320	0	5120	640	160	0	0
O8	40	0	20	0	1280	160	80

O antigens of strain Ar 122: O11a,11c; unilateral relationship to O8

2. Antigenic changes after lysogenization

Several of the lysogenized strains produced R-type colonies and some mucoid colonies were also encountered. The derivatives dealt with in this paper were characterized by normal colonial morphology and heat-stable suspension. Although all 23 O antigen type strains were lysogenized by all 22 phages, only strains O1, O4a,4b and O4a,4c split off such kinds of derivatives. These cultures usually differed from the parent strain in agglutinating in different group sera prepared with unrelated antigens and having the capacity of considerably decreasing the titres of the corresponding heterologous sera. Many of the derivatives showed unstable antigenic alterations and upon subsequent streakings and single colony isolations they reverted to the original antigenic structure. One unstable derivative, obtained by lysogenization of strain 170001 (O1) by phage F8, resembled closely in antigenic structure to culture Lak 744 isolated from infants (O antigen identical with O1, unilateral relationship to O8). Derivatives representing the different patterns of stable antigenic changes were subjected to detailed analysis (Tables III–VI).

In view of a possibility of a transduction of O antigens from the propagating strains to the lysogenized derivatives, the antigenic structure of the former was also determined (see headlines for Tables III–VI). Although in this respect there was no definite correlation between the propagating and derivative cultures, it is interesting that the majority of phages found responsible for conversion to multiple agglutinability originated from strains belonging to serogroups O1 (strain 68), O4 (strains 73, F8, 119x, C11), O8 (strain 21) and O10 (strains 2 and M4). The propagating strains for phages 16, 44 and 1214, also converting to multiple agglutinability, contained antigens not showing the slightest relationship to antigens of the lysogenized derivatives: O3 (strains 44 and 1214), O7 (strain 16).

Derivative 8/2 (Table III). The isolate reacted in sera O1, O8, O10a and O11 at considerably higher titres than the parent strain; the serum prepared with the derivative agglutinated strains O1, O8 and O11, but not O10a. Cross-absorption experiments showed that the culture removed practically all agglutinins from the serum for the parent strain. The presence of antigen O4a is evident from cross-agglutination in sera O4a,4b, O4a, 4c and O4a,4d; O4b content is proved by the full titre reaction in serum O4a,4b absorbed by O4a,4c.

Derivative 8/C18 (Table IV). The culture differed from derivative 8/2 in giving bilateral cross reaction not only with O1, O8 and O11 but also with O10a; the absorption experiments proved this difference: whereas type strain O10a practically failed to remove agglutinins from serum 8/2, it decreased the titre of serum 8/C18 considerably.

Table III

*Antigenic structure of P. aeruginosa derivative 8/2*Parent strain, *P. aeruginosa* O4a,4b (170008) lysogenized with phage 2 (propagating strain, *P. aeruginosa* O10a)

Strain	Serum												
	O1		O4a,4b			O4a,4c			O4a,4d			O8	
	unabs.	abs. by 8/2	unabs.	abs. by O4a,4c	abs. by 8/2	unabs.	abs. by O4a,4b	abs. by 8/2	unabs.	abs. by O4a,4c	abs. by 8/2	unabs.	abs. by 8/2
O1	2560	2560	20	0	0	0	0	0	0	0	0	20	0
O4a,4b	40	0	1280	320	20	1280	0	40	160	0	0	40	0
O4a,4c	0	0	640	0	0	1280	640	20	640	0	0	40	0
O4a,4d	0	0	160	0	0	160	0	0	2560	640	160	0	40
O8	40	0	0	0	0	0	0	0	40	0	0	1280	40
O10a	40	0	0	0	0	0	0	0	40	0	0	40	0
O11	80	0	40	0	0	0	0	0	0	0	0	0	0
8/2	320	0	640	320	0	640	320	0	640	0	0	320	0

Strain	Serum											
	O10a		O11		8/2							
	unabs.	abs. by 8/2	unabs.	abs. by 8/2	unabs.	abs. by O1	abs. by O4a,4b	abs. by O4a,4c	abs. by O4a,4d	abs. by O8	abs. by O10a	abs. by O11
O1	20	0	40	0	160	0	0	0	0	0	0	0
O4a,4b	20	0	40	0	5120	5120	0	640	320	0	80	160
O4a,4c	20	0	40	0	1280	1280	20	0	40	20	80	80
O4a,4d	0	0	80	0	160	80	0	0	0	0	0	0
O8	40	0	40	0	160	0	0	0	0	0	0	0
O10a	1280	640	40	0	20	0	0	0	0	0	0	0
O11	80	0	1280	1280	640	0	0	0	0	0	0	0
8/2	320	0	160	0	10240	5120	640	5120	5120	2560	5120	5120

O antigens of derivative 8/2: O4a,O4b; bilateral relationship to O1, O8 and O11, unilateral relationship to O10a

Table IV

*Antigenic structure of P. aeruginosa derivative 8/C18*Parent strain, *P. aeruginosa* O4a,4b (170008) lysogenized with phage C18 (propagating strain, *P. aeruginosa* O8)

Strain	Serum												
	O1		O4a,4b			O4a,4c			O4a,4d			O8	
	unabs.	abs. by 8/C18	unabs.	abs. by O4a,4c	abs. by 8/C18	unabs.	abs. by O4a,4b	abs. by 8/C18	unabs.	abs. by O4a,4c	abs. by 8/C18	unabs.	abs. by 8/C18
O1	2560	1280	20	0	0	0	0	0	0	0	0	20	0
O4a,4b	40	0	1280	320	20	1280	0	0	160	0	0	40	0
O4a,4c	0	0	640	0	0	1280	640	0	640	0	0	40	0
O4a,4d	0	0	160	0	0	160	0	160	2560	640	160	40	0
O8	40	0	0	0	0	0	0	0	40	0	0	1280	320
O10a	40	0	0	0	0	0	0	0	40	0	0	40	0
O11	80	0	40	0	0	20	0	0	0	0	0	0	0
8/C18	320	0	640	320	0	640	0	0	640	0	0	320	0

Strain	Serum											
	O10a		O11		8/C18							
	unabs.	abs. by 8/C18	unabs.	abs. by 8/C18	unabs.	abs. by O1	abs. by O4a,4b	abs. by O4a,4c	abs. by O4a,4d	abs. by O8	abs. by O10a	abs. by O11
O1	20	0	40	0	160	0	0	40	0	0	0	0
O4a,4b	20	0	40	0	2560	640	0	80	80	80	640	640
O4a,4c	20	0	40	0	640	320	0	0	0	0	640	640
O4a,4d	0	0	80	0	640	160	0	0	0	0	0	0
O8	40	0	40	0	320	0	0	0	0	0	0	0
O10a	1280	320	0	0	640	0	0	320	160	0	0	0
O11	160	0	1280	160	160	0	0	80	80	0	0	0
8/C18	640	0	640	0	5120	640	80	2560	2560	640	320	320

O antigens of derivative 8/C18: practically identical with O4a and O4b; bilateral relationship to O1, O8, O10a and O11

Derivative 9/73 (Table V). Neither O4a nor O4c antigens proved to be identical in the parent strain and in the derivative. The close relationship of the derivative to antigen O8 and traces of connection to antigens O10a and O11 are evident.

From Tables III, IV and V it may be concluded that the antigenic differences between the three derivatives are rather quantitative than qualitative and that the antigens of the parent strains are, at least partially, present in all three derivatives.

Derivative 8/1214. A lysogenic conversion from one antigenic group to another is shown in Table VI. The derivative exhibited but a low titre agglutination in the serum for the parent strain and *vice versa*. A high titre cross-agglutination was demonstrated between the derivative and type strains O10a and O10a,10b. Cross-absorption with the latter showed that the isolate contained complete antigen O10a, whereas antigen O10b was absent. From Table VI it is, however, evident that the derivative differed from type strain O10a in showing a minor unilateral relationship to O1.

The fact that derivative 8/1214 was the only one showing conversion from one serogroup to another, suggested the possibility of an experimental error. As lysogenized type strain O4a,4b and type strain O10a (the most probable origin of 8/1214 if contamination had occurred) differed in flagellar antigens (H2a,2b and H1, respectively), it was expected that determination of the H antigen of derivative 8/1214 would settle the problem. Other markers were of no use in tracing the origin of 8/1214, as in 105 different biochemical and cultural characters strains O4a,4b and O10a behaved uniformly. The derivative at first failed to agglutinate in H sera, but after several subcultures in nitrate semisolid agar, it developed motility and showed typical flagellar agglutination in serum H1. The probability of an experimental error was also suggested by the fact that repeated lysogenization experiments failed to reproduce derivatives agglutinating in serum O10a but not in serum O4a,4b.

3. *Immunoelectrophoretic pattern of parent strains and their derivatives*

Immunoelectrophoresis of different extracts of the cultures showed that the parent strains and their derivatives belonged to the same immunoelectrophoretic group (Table VII). The only exception was derivative 8/1214, which gave a pattern characteristic of strains containing antigen O10 [3].

4. *Phage typing and prophage induction*

Phage sensitivity pattern of parent strains and their derivatives. Table VIII shows the change in phage sensitivity pattern after lysogenization. The alteration in antigenic structure was usually accompanied by a loss of sensitivity to several phages.

Table V

*Antigenic structure of P. aeruginosa derivative 9/73*Parent strain, *P. aeruginosa* O4a,4c (170009) lysogenized with phage 73 (propagating strain, *P. aeruginosa* O4a,4c)

Strain	Serum												
	O1		O4a,4b			O4a,4c			O4a,4d			O8	
	unabs.	abs. by 9/73	unabs.	abs. by O4a,4c	abs. by 9/73	unabs.	abs. by O4a,4b	abs. by 9/73	unabs.	abs. by O4a,4c	abs. by 9/73	unabs.	abs. by 9/73
O1	2560	1280	20	0	0	0	0	0	0	0	0	20	0
O4a,4b	40	0	2560	320	320	1280	0	80	160	0	20	40	0
O4a,4c	0	0	640	0	40	1280	640	80	640	0	320	40	0
O4a,4d	0	0	160	0	80	160	0	0	2560	640	2560	40	0
O8	40	40	0	0	0	0	0	0	40	0	0	1280	20
O10a	40	40	0	0	0	0	0	0	40	0	0	40	0
O11	80	0	40	0	0	20	0	0	0	0	0	0	0
9/73	160	0	320	0	0	1280	80	0	80	0	0	1280	0

Strain	Serum											
	O10a		O11		9/73							
	unabs.	abs. by 9/73	unabs.	abs. by 9/73	unabs.	abs. by O1	abs. by O4a,4b	abs. by O4a,4c	abs. by O4a,4d	abs. by O8	abs. by O10a	abs. by O11
O1	20	0	40	0	640	0	0	0	0	0	80	160
O4a,4b	20	0	40	0	160	0	0	80	0	80	80	40
O4a,4c	20	0	40	0	160	0	0	0	0	0	80	80
O4a,4d	0	0	80	0	160	0	0	160	0	0	80	80
O8	40	0	40	0	40	0	0	0	0	0	0	0
O10a	1280	640	40	0	160	0	0	0	0	0	0	80
O11	80	0	1280	640	160	0	0	0	0	0	80	0
9/73	40	0	20	0	1280	0	0	0	0	160	640	640

O antigens of derivative 9/73: close bilateral relationship to O4a and O4c; bilateral relationship to O1; marked unilateral relationship to O8; weak unilateral relationship to O10a and O11

Table VI*Antigenic structure of P. aeruginosa derivative 8/1214*Parent strain, *P. aeruginosa* O4a,4b (170008) lysogenized with phage 1214
(propagating strain, *P. aeruginosa* O3a,3d,3e)

Strain	Serum					
	O1		O4a,4b	O10a		
	unabs.	abs. by 8/1214	unabs.	unabs.	abs. by O10a,10b	abs. by 8/1214
O1	2560	2560	20	20	0	0
O4a,4b	40	0	1280	20	0	0
O10a	40	0	0	1280	0	0
O10a,10b	40	0	0	1280	0	0
8/1214	320	0	160	2560	0	0

Strain	Serum					
	O10a,10b			8/1214		
	unabs.	abs. by O10a	abs. by 8/1214	unabs.	abs. by O10a	abs. by O10a,10b
O1	0	0	0	0	0	0
O4a,4b	0	0	0	80	0	0
O10a	640	0	0	1280	0	0
O10a,10b	2560	640	640	5120	0	0
8/1214	1280	0	0	5120	0	0

O antigens of derivative 8/1214: identical with O10a; unilaterally related to O1

Lytic spectra of phages liberated from lysogenized derivatives. As shown in Table IX, phages released from the derivatives after UV irradiation differed in lytic spectrum from the infecting phages. The lytic spectrum usually became narrower and the titre of UV-induced phages was considerably lower than that of the infecting phages. It should be noted that parent strains 170001 and 170008 released phages (even without UV irradiation) which acted on some of the indicator strains.

5. Stability of the derivatives

Subculture on nutrient media. The parent strains and their derivatives were subjected to serial subculture on nutrient agar and on blood agar plates by starting on each day from single colonies. After 30 subcultures there was no appreciable change in colonial morphology, antigenic structure, phage sensitivity and prophage production. The only exception was derivative O1,

Table VII

Characteristics of P. aeruginosa parent strains and their derivatives examined in this study

Source of derivative	Parent strain				Derivative			
	Designation	O antigens	H antigens	IE group	Designation	O antigens	H antigens	IE group
Infants	—*	<i>1</i>	<i>1</i>	Ib	Lak 744	<i>1,(8)</i>	<i>1</i>	Ib
	—**	<i>11a,11b</i>	<i>2a,2c,2f</i>	IV	Ar 122	<i>11a,11c(8)</i>	<i>2a,2c,(2f)</i>	IV
Lysogenization <i>in vitro</i>	170001	<i>1</i>	<i>1</i>	Ib	1/F8	<i>1,(8)</i> unstable	<i>1</i>	Ib
					8/2	<i>4a,4b,1,</i> <i>8,(10a),11</i>	<i>2a,2b</i>	II
	170008	<i>4a,4b</i>	<i>2a, 2b</i>	II	8/C18	<i>4a,4b,1,</i> <i>8,10a,11</i>	<i>2a,2b</i>	II
					8/1214	<i>10a,(1)</i>	<i>1</i>	Ic
	170009	<i>4a,4c</i>	NM	Ic	9/73	<i>4a,4c,1,(8)</i> <i>(10a),(11)</i>	NM	Ic

* Probable parent organism: *P. aeruginosa* O1** Probable parent organism: *P. aeruginosa* O11

IE group = immunoelectrophoretic group

Italicized figures: antigenic identity with O antigens of the type strains; non-bracketed figures: bilateral relationship; bracketed figures: unilateral relationship

(O8) which either reverted to pure O1 form or became agglutinable after a few passages not only in sera O1 and O8 but also in O4, O10 and O11. Strain Lak 744, a natural O1, (O8) variant had a similar tendency of becoming agglutinable in the above sera with a simultaneous change in colonial morphology; by selecting the proper colonies, however, its O1, (O8) form has been maintained.

Freeze-drying. After freeze-drying in rabbit serum-CI suspending fluid and storage of the ampoules for 1 year at 18–20 °C, there was no change in colonial morphology, antigenic structure, phage sensitivity and prophage production.

Table VIII

Phage sensitivity pattern of parent strains and their lysogenized derivatives

Culture	Phage sensitivity pattern	Changes in phage sensitivity pattern		Lyso- genicity*
		+	-	
70001 parent	2/7/44/68/F8/109/352/1214/C18			+
1/F8 derivative	2/7/109/C18		44/68/F8/352/1214	+
1170008 parent	2/7/21/44/F8/109/352/1214/C18			+
8/2 derivative	21/C188	C188	2/7/44/F8/109/352/1214/C18	+
8/C18 derivative	21/C188	C188	2/7/44/F8/109/352/1214/C18	+
8/1214 derivative	2/7/21/44/F8/109/119x/C11/C18	119x/C11	352/1214	+
170009 parent	2/21/73/119x/352/M4/C11/C18			-
9/73 derivative	2/21/31/352/C18/C188	31/C188	73/119x/M4/C11	+

* Activity of supernatants of UV-induced cultures on propagating strains of the standard phage set

Table IX

Comparison of the lytic spectrum of standard typing phages and of phages released from the parent strains and lysogenized derivatives

Infecting phage	Phage released from		Lytic spectrum on propagating strains	Change in lytic spectrum as compared to infecting phage	
	parent strain	lysogenized derivative		+	-
F8	170001	1/F8	2/M4/M6 2/24/68/F8/352/M4/C11/C18/C21 2/M4/(M6)	(M6)	24/68/F8/352/C11/C18/C21
2	170008	8/2	2/F8/M4/(M6)/C11 2/68/F8/352/M4/C11/C18 2/68/F8/M4/(M6)	(M6)	352/C11
C18		8/C18	2/F7/119x/1214/M4/M6/C11/C18 68/(F7)/M4/C11/C18	68	2/119x/1214/M6
1214		8/1214	2/24/44/68/F8/1214/M4/M6/C11/C18/C21 68/F8/M4/(M6)		2/24/44/1214/C11/C18/C21
73	170009	9/73	2/73/M4 (73/M4)		2

Discussion

Lysogenization of 23 *P. aeruginosa* O antigen type strains with 22 different phages resulted in a conversion of one serogroup into another only in one case in which, however, the possibility of an experimental error could not reliably be excluded. The rest of the lysogenized derivatives contained O antigens of the parent strains in addition to factors bilaterally or unilaterally related to other O groups. Type strains O1, O4a,4b and O4a,4c were the only ones that split off such antigenic variants after lysogenization.

The possibility of antigen transduction may be considered in view of the fact that most of the phages converting to multiple agglutinability had been propagated on strains containing O factors appearing, among others, in the derivatives. Transduction at the level of complete O antigens, however, can be excluded, since definite correlation between the antigenic structure of the derivative and of the propagating strain has never been demonstrated, e.g. antigen O10a was a minor factor in derivative 8/2 (the infecting phage for which had been propagated on strain O10a), whereas the same antigen was a prominent factor in derivative 8/C18 (the corresponding propagating strain for which contained antigen O8).

Multiple agglutinability of the lysogenized derivatives was proved in different batches of sera O1, O4, O8, O10 and O11 both with slide agglutination of living, and with tube agglutination of heated cultures. Slide agglutination frequently appeared as a loose flaky aggregation of bacteria instead of a granular clumping characteristic of group-specific O agglutination. As the derivatives retained their smooth colonial form and their suspensions remained heat-stable, the change may be interpreted as a degree of serological R variation or degradation. This assumption is supported by the fact that all derivatives were related to antigen O8, which itself is an R-like factor [3-5].

Antigenic variations supposed to have taken place in infants were also characterized by a partial change in antigenic structure. One of the isolates, strain Ar 122(O11a,11c), in view of the fact that it was isolated from natural source, is stable S-form morphologically and serologically and belongs to a distinct serogroup, should be regarded as representing a new subgroup of the *P. aeruginosa* antigenic schema.

As obvious from literary data, many authors have not distinguished between multiple agglutinability which occurs in certain sera, and spontaneous agglutinability which takes place in any serum as well as in serum-free saline. Testing heated *P. aeruginosa* suspensions by tube agglutination, HOMMA *et al.* [6] described the incidence of multiple agglutinability as 15.5%, whereas with slide test LÁNYI [1], LÁNYI *et al.* [7] and NÉMEDI and LÁNYI [8] showed such cultures in 0-13.0%. Strains agglutinating in saline are less common in

nature; from the observations of HOMMA *et al.* and of LÁNYI *et al.* their incidence may be estimated at 0–1.0%.

According to our early observations on isolates from a wide variety of sources, multiple agglutination occurs as a rule in group sera O1, O4, O8, O10, O11 and occasionally in O9. In fact, this is the main reason why polyvalent serum OI has been pooled so as to contain agglutinins for O1, O4, O8, O9, O10 and O11 [1]. In a recent study by KATONA and LÁNYI [9] on the epidemiology of *P. aeruginosa* mastitis in cows, a number of antigenic variants have been isolated from the environment of herds infected predominantly with serogroups O1, O4a,4c and O4a,4d. These isolates were also very similar in antigenic structure to those obtained after lysogenization of O4a,4b and O4a,4c strains: they showed multiple agglutination in sera O1, O4, O8, O10 and O11.

Changes in O antigenic structure of *P. aeruginosa* have been described by several authors. HOLLOWAY and COOPER [10] were the first to observe an a, b-a, c-type conversion after lysogenization *in vitro*. LÁNYI [1] described a change assumed to have occurred in an intensive care unit, where a patient after long-term harbouring of *P. aeruginosa* serogroup O7, began to excrete a serovariant characterized by formula O7a,7c whereas the original cultures contained antigens O7a,7b.

LIU [11] performed a large number of lysogenization experiments with *P. aeruginosa* and described several antigenic changes. BERGAN and MIDTVEDT [12] produced for the first time the same change by lysogenization *in vitro* and in germ-free rats contaminated with *P. aeruginosa* then fed with phage. It is interesting that BERGAN and MIDTVEDT starting from serogroup Habs 5 (LÁNYI O3a, 3d, in which we failed to induce antigenic alteration by lysogenization) showed a change to serogroups Habs 6 and 9, the corresponding antigens of which (LÁNYI O4a, . . . and O10a) were present in our derivatives, too. Our finding that the lysogenized derivatives exhibited a narrower phage sensitivity pattern than the parent strains, are in agreement with the results of BERGAN and MIDTVEDT. The lytic spectrum of phages released from the derivatives after UV induction fairly differed from the lytic spectrum of the corresponding parent culture. This was not surprising, as the original phage had reached a high titre on its own propagating strain, whereas the prophages were of relatively low titre.

HOMMA *et al.* [13] and KAWAHARAJO [14] described antigenic changes taking place after long-term preservation of *P. aeruginosa* cultures on nutrient agar. In the present experiments serial subculturing and storing caused changes in some of our derivatives but not in the O antigen type strains. BERGAN and MIDTVEDT [12] described that after lyophilization the lysogenized cultures had reverted to the original serogroup and to the original phage sensitivity

pattern and lost their prophage-producing ability. In contrast, our lysogenized derivatives failed to show any alteration after freeze-drying.

To conclude, it may be assumed on the basis of epidemiological evidence that antigenic changes characterized by retaining part of the original antigen and acquiring a new factor, that is, a, b-a, c alterations, occur occasionally in nature. In contrast, change to multiple agglutinability takes place frequently and is probably associated with phage action.

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IMMUNITY OF THE GUINEA PIG'S EYE AFTER VACCINATION WITH SHIGELLA SONNEI PHASE I ANTIGEN

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Summary. A total of 8 doses, each containing 10^9 live or heated cells of pure phase I antigen of *Shigella sonnei* and *Plesiomonas shigelloides*, was administered into the conjunctival sac of guinea pigs during a 3-week period. The eyes were challenged with virulent *S. sonnei*. The vaccines protected the eyes in 50-80% to the minimum infecting dose and in 20-50% to 3 MID. The peak of immunity began to decrease after the 26th day and on the 56th day it was practically nil. Vaccination caused histological changes in the conjunctiva but not in the cornea.

Literary data reviewed by SERÉNY *et al.* [1] show that live *Shigella flexneri* vaccine has been used successfully. Preparation of live *Shigella sonnei* vaccine presents difficulties since phase I $\rightarrow$ II variation causes a loss of immunizing potency against virulent organisms. In the present experiments the immunizing capacity of avirulent *S. sonnei* variants with stable phase I antigen and of *Plesiomonas shigelloides* containing the same antigen was examined with SERÉNY's guinea pig eye test [2].

Materials and methods

Vaccines. *S. sonnei* cultures freshly isolated from patients were subcultured in peptone water. After the fifth passage avirulent phase I colonies appeared. On agar plates examined at oblique light or under the phase microscope they were characterized by golden-green colour, homogeneous or slightly granular structure similar to *S. flexneri* colonies and were easily distinguished from virulent phase I + II or avirulent phase II forms [3-5]. *P. shigelloides* cultures containing *S. sonnei* phase I antigen were isolated from surface water. The cultures were checked for avirulence by the guinea pig eye test [6].

The following vaccines were prepared: (a) 24 hr agar plate culture; (b) 24 hr Roux flask agar culture suspended in saline; (c) 24 hr Roux flask agar culture dried at 5 °C *in vacuo* then suspended in saline; (d) saline suspension heated at 100 °C for 2 hr.

Immunization and challenging. Both eyes of guinea pigs weighing approximately 300 g were immunized three times weekly with a total of 8 doses each containing 10^9 bacterial cells (one loopful of agar plate culture or 0.02 ml suspension). One week after the last dose the eyes were challenged with 1-3 minimum infecting doses (MID) of virulent *S. sonnei*. Eyes showing no evidence of keratoconjunctivitis 1 week after the challenge were recorded as effectively immunized. The same number of non-vaccinated eyes were used as control. Groups of animals immunized with *S. sonnei* phase II and groups having recovered from keratoconjunctivitis shigellosa elicited by virulent organisms were also tested.

Histological examination was performed at 1, 17 and 24 hr intervals after vaccination then 1 week after the last dose and finally 2 and 10 days after challenge, if keratoconjunctivitis had not developed. The eyeball was halved by a sagittal section and embedded into celloidin-paraffin. Sections 4-5 μ m in thickness were stained with haematoxylin-eosin and thionin.

Results

Immunogenicity. The organisms inoculated onto the conjunctiva remained viable for days. The number of colonies recovered decreased gradually; a few colonies appeared 3–4 days after live *S. sonnei* I and 2–3 days after live *S. sonnei* II vaccination. *P. shigelloides* was usually recovered only in the first 24 hr (10–20 S-form colonies or 0–10 R-form colonies).

Table I shows a comparison of the immunogenicity of live avirulent *S. sonnei* and *P. shigelloides* and their corresponding R-forms. The S-form vaccines afforded an identical degree of protection (26–29%), whereas the phase II and R variants failed to exert any immunogenicity. In guinea pigs which had recovered from keratoconjunctivitis shigellosa, the immunity was more marked (60%) than after immunization with S-form vaccines.

Table I
Immunogenicity of live vaccines
Challenging dose: 3 MID virulent *S. sonnei*

Groups of animals	Number of guinea pig eyes		
	Total	Positive*	Negative**
<i>S. sonnei</i> I 384/2	38	28	10
<i>P. shigelloides</i> 266 S	34	24	10
<i>S. sonnei</i> II	38	38	—
<i>P. shigelloides</i> 266 R	40	40	—
Not vaccinated	34	34	—
Previous keratoconjunctivitis shigellosa	40	15	25

* No. of eyes developing keratoconjunctivitis

** No. of symptomless eyes

Table II shows the time course of immunity developing after vaccination with live *S. sonnei* I. On the 24th day postvaccination the immunity was at its peak, whereas on the 54th day it was practically nil. The data for the three groups of animals challenged with virulent *S. sonnei* were analysed statistically comparing the eyes with and without keratoconjunctivitis at different intervals after immunization. Two separate comparisons were made: (a) between the immunized and non-immunized control groups; (b) between animals with previous keratoconjunctivitis and control animals. The results were analysed in 2×2 contingency tables and examined for significance by the χ^2 test at the 99.9% probability level. When the frequency of random variables was lower than the number required, the trend of difference was estimated.

Table II

Time course of immunity in guinea pig eyes vaccinated with live S. sonnei I strain 384/3
Challenging dose: approx. MID virulent *S. sonnei*

Days after immunization	Challenging dose	Immunized eyes			Non-immunized eyes			Eyes with previous keratoconjunctivitis		
		No.	+	-	No.	+	-	No.	+	-
4	4×10^8	36	18*	18	36	36	—	36	4*	32
12	1.6×10^8	36	18*	18	36	35	1	36	5*	31
26	8×10^7	36	11*	25	36	33	3	36	5*	31
54	1.2×10^8	34	31	3	36	36	—	36	9*	27

+ No. of eyes developing keratoconjunctivitis

— No. of symptomless eyes

* Mostly mild keratoconjunctivitis

(a) The difference between the immunized and control groups was significant 4 days ($\chi^2_{[1]} = 22.685$, $p < 0.001$), 12 days ($\chi^2_{[1]} = 20.663$, $p < 0.001$) and 26 days ($\chi^2_{[1]} = 28.286$, $p < 0.001$) after immunization. The trend of difference was not significant 54 days after immunization.

(b) The difference between animals that had recovered from keratoconjunctivitis and the control animals was significant not only at the 4th ($\chi^2_{[1]} = 57.600$, $p < 0.001$), 12th ($\chi^2_{[1]} = 37.137$, $p < 0.001$) and 26th ($\chi^2_{[1]} = 43.690$, $p < 0.001$) but also at the 54th ($\chi^2_{[1]} = 43.200$, $p < 0.001$) day interval. Accordingly, previous infection with virulent cultures yielded an immunity lasting considerably longer than that elicited by immunization with avirulent vaccine.

The two kinds of immunity differed in the number of eyes resistant to the challenging dose: (a) in the immunized group the 4, 12, 26 and 54 day values were 50, 50, 69 and 9% respectively, whereas (b) in the group with a history of keratoconjunctivitis the corresponding percentages were 89, 86, 86 and 75.

These findings indicate that recovery from keratoconjunctivitis results in immunity which is more effective and permanent than that developing after vaccination with live avirulent *S. sonnei* I.

As the technique of conjunctival infection makes it difficult to apply standard challenging doses, a separate analysis was performed to compare the results in each group 12 and 54 days after immunization; the challenging doses were 1.6×10^8 and 1.2×10^8 , respectively. In the immunized group the difference was significant ($\chi^2_{[1]} = 14.118$, $p < 0.001$). In the control group the ratio of positive eyes was 97.2% in the 12 day subgroup and 100% in the 54 day subgroup; animals which had recovered from keratoconjunctivitis showed no significant difference ($\chi^2_{[1]} = 1.419$, $p > 0.05$).

In subsequent experiments two *S. sonnei* I vaccine strains cultured on different media were compared (Table III). The two strains exerted an identical protective effect and the desoxycholate or desoxycholate + citrate content of the medium did not influence the results. Three additional strains (*S. sonnei* I Nos 110, 629 and 928) behaved similarly.

Table III

Immunogenicity of different S. sonnei I vaccines
Challenging dose: 9.6×10^7 virulent *S. sonnei*

Strain	Culture medium	Number of guinea pig eyes		
		Total	Positive*	Negative**
384/I	NA	30	11	19
	D	30	18	12
	DC	30	12	18
1850	NA	30	6	24
	D	10***	7	3
	DC	28	10	18
Control group		30	29	1

* No. of eyes developing keratoconjunctivitis

** No. of symptomless eyes

*** The rest of the animals died

NA = nutrient agar

D = desoxycholate agar

DC = desoxycholate citrate agar

Table IV

Immunity of vaccinated guinea pig eyes to different challenging doses

Strain	Culture medium	Challenging dose	Number of guinea pig eyes		
			Total	Positive*	Negative**
1850	NA	approx.	30	5	25
1850	EMB	MID	26	10	16
Control group			30	29	1
1850	NA	3 MID	28	16	12
1850	EMB	3 MID	28	16	12
Control group			30	30	—

* No. of eyes developing keratoconjunctivitis

** No. of symptomless eyes

NA = nutrient agar

EMB = eosin methylene blue agar

Table IV shows that the resistance of the eyes is proportional to the challenging dose (the same is evident from the comparison of data in Tables I, II and III).

The effect of repeated immunization was also examined. Immunized eyes not developing keratoconjunctivitis were reimmunized (Table V). There was no increase in immunity.

Table VI shows that, at least for the lowest infecting dose, heated vaccines exerted an immunogenicity similar to that of live vaccines. *S. sonnei* vaccines failed to protect the eyes against *S. flexneri* infection.

Table V

Immunity of guinea pig eyes after repeated vaccination
Second immunization: 60 days after first vaccination

Days after second immunization	Challenging dose	No. of eyes after second immunization			No. of non-immunized eyes		
		Total	Positive*	Negative**	Total	Positive*	Negative**
7	9.2×10^7	36	17***	19	36	34	2

* No. of eyes developing keratoconjunctivitis

** No. of symptomless eyes

*** Mostly mild keratoconjunctivitis

Table VI

Immunogenicity of vaccines prepared in different manners
Challenging dose: somewhat less than MID

Vaccine	Number of guinea pig eyes		
	Total	Positive*	Negative**
24 hr live	30	1	29
Freshly dried	30	15	15
Dried and stored for 6 months	30	9	21
Heated at 100 °C for 2 hr	30	9	21
Control group	30	26	4

* No. of eyes developing keratoconjunctivitis

** No. of symptomless eyes

Histological examination. One hour after inoculation with live culture 384/I, leukocytic infiltration, microabscesses and bacteria were observed in the conjunctival epithelium. By the 16th and 24th hr there was a slight leukocytic reaction and the bacteria had disappeared. The cornea remained intact throughout the experiment.

After the end of immunization, either *S. sonnei* or *P. shigelloides* had been given, there was a marked secretion with a moderate chronic inflammatory infiltration in the conjunctival epithelium and connective tissue. In the canthal lymph nodule a marked lymphoreticular activity appeared.

On the 1st, 2nd and 7th days after challenge with the virulent organism, the macroscopically symptomless eyes showed a mild leukocytic infiltration and activity in the canthal lymphoreticular nodule; bacteria and injury of the cornea were absent.

Discussion

Vaccines containing *S. sonnei* phase I antigen produce local immunity in the guinea pig's eye against infection with virulent *S. sonnei*. Vaccines prepared from *S. sonnei* phase II and from the R-form of *P. shigelloides* were ineffective. The development of immunity depends probably only on the contact with the specific antigen, since the type of vaccine (*S. sonnei* I, *P. shigelloides*, live or heated) was indifferent. The difference between vaccination and a previous infection with virulent shigellae is evident from the fact that the latter establishes a more effective and more permanent immunity.

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THE ROLE IN SPORADIC ENTERITIS OF FACULTATIVELY ENTEROPATHOGENIC ESCHERICHIA COLI SEROGROUPS

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Summary. During a two-year period, 2411 faecal specimens and 514 throat swabs were cultured from 1674 persons, mainly infants and children. Of 31 *Escherichia coli* serogroups considered to represent facultatively enteropathogenic agents, the following were encountered: O4, O18a,b, O18a,c, O19, O20, O25, O28a,b, O32, O51, O53, O75, O78, O79, O105, O112a,c, O114, O115, O117, O129, O143, O145, O148. There was no significant difference in the incidence of the organisms and their serogroup distribution between patients with enteritis, patients with extraintestinal diseases and healthy individuals. A rise of antibody titre in some of the patients indicated an association with enteritis in case of serogroups O4, O18a,c, O19, O25, O75, O78 and O117. It has been assumed that the presence in faeces of a facultatively pathogenic *E. coli* serogroup does not necessarily prove its aetiological relationship to the disease.

Since the establishment of the *Escherichia coli* antigenic schema, 42 serotypes have been described as causative agents of enteric diseases [1–4]. Although recently further serogroups have been added to the list [5, 6] the aetiology of a high proportion of enteric infections still remains undetected [7–10] and the obscure pathomechanism of *E. coli* enteritis [11–22] also presents diagnostic problems.

Since 1970 we have been collecting data for certain *E. coli* serogroups described in the literature and in our own papers [23–27] as capable of causing enteritis in addition to recognized enteropathogenic *E. coli* serogroups. The purpose of the present study was to establish the incidence of these organisms, to be termed “facultatively enteropathogenic” *E. coli* serogroups, in faecal specimens from a variety of sources and to seek answers to the following questions: (i) do facultatively enteropathogenic *E. coli* serogroups occur more frequently in patients with enteritis than in other patients? (ii) does the isolation of these bacteria prove their aetiological role in enteritis?

Materials and methods

Groups of persons. (i) Mainly infants with enteritis, treated at László Hospital for Infectious Diseases. Total, 495; age distribution of patients with positive bacteriological finding (years): 0–1, 149; 1–3, 15. (ii) Infants and children, mostly with extraintestinal diseases, treated at Semmelweis Hospital. Total, 120; age distribution (years): 0–1, 40; 1–3, 35; 4–6, 31; 7–14, 14. (iii) Persons with sporadic enteritis, Százhalombatta town; total 249; age distribution (years): 0–1, 52; 1–3, 84; 4–6, 30; 7–14, 40; above 14, 43. (iv) Persons in the environment of the latter; total, 625; age distribution (years): 0–1, 24; 1–3, 41; 4–6, 52; 7–14, 56; above 14, 452. (v) Healthy persons screened for enteric pathogens in Pest county: total, 185; age distribution (years): 0–1, 72; 1–3, 45; 4–6, 18; 7–14, 7; above 14, 43.

Specimens. Faeces, throat swabs, blood serum.

Isolation of bacteria. All morphologically different coliform colonies grown on eosin methylene blue agar at 37 °C for 18 hr were transferred to agar slants. Pathogenic enterobacteria (salmonellae, shigellae, *E. coli* O26:K60, O55:K59, O86:K61, O111:K58, O112:K66, O119:K69, O124:K72, O125:K70, O126:K71, O127:K63, O128:K67) were isolated and identified as specified in the Standard Methods of Hungarian Public Health Bacteriology Laboratories [28].

Preparation of E. coli O sera. International type strains for groups O4, O13, 18a,b, O18a,c, O19, O20, O25, O28a,b, O28a,c, O32, O51, O53, O58, O73, O75, O78, O79, O105, O112a,b, O112a,c, O114, O115, O117, O129, O135, O136, O142, O143, O144, O145 and O148 were used. The suspensions were heated at 100 °C for 2½ hr and injected into rabbits as described by EDWARDS and EWING [29]. The sera were tested for cross-agglutination with heated suspensions of *E. coli* type strains O1–O163 by single tube technique using serum dilutions 3–4 times more concentrated than the highest dilution still agglutinating the homologous culture (e.g. 1 : 1000 vs. 1 : 25 600). Most sera reacted with 2–3 different serogroups; sera O4, O13, O18, O19, O25, O73, O129, O135 and O142 agglutinated 12–15 different antigens. The heterologous reactions were eliminated by absorbing the sera with all reacting antigens. The four pooled sera were composed on the basis of the cross-agglutination patterns.

Serogrouping of the isolates. Agar slant cultures grown at 37 °C for 18 hr were suspended in 5 ml physiological saline, adjusted to contain 10⁹ cells/ml and heated at 100 °C for 2½ hr. The suspensions were added at 0.05 ml amounts to 0.5 ml diluted pooled and group sera. The isolates were considered to belong to a defined O group if agglutination occurred in one of the adsorbed O sera to the titre of the homologous type strain.

Passive haemagglutination. Serum antibodies for *E. coli* serogroups harboured by the patient were detected by passive haemagglutination using TAKÁTSY's Microtitrator set. Two parallel dilutions were prepared from each serum specimen and tested with sheep erythrocytes sensitized with *E. coli* antigens as described by WINBERG *et al.* [30] and NETER *et al.* [31]. The highest dilution showing ++++ haemagglutination was regarded as the end-point. Each serum sample was checked with non-sensitized erythrocytes. Sera from infants with enteritis not harbouring the serogroup under investigation were used as negative controls (<1 : 16). Hyperimmune rabbit sera prepared with the homologous antigen served as positive control: different batches of the sensitized erythrocytes were standardized so as to give the same titre in the hyperimmune serum.

Statistical analysis. The data were analysed in 2 × 2 contingency tables and examined for significance by the χ^2 test [32].

Results

Infants with enteritis, László Hospital. Table I shows the incidence of enteropathogenic and facultatively enteropathogenic agents in infants admitted

Table I
Incidence of pathogenic Enterobacteriaceae and

Groups of persons	No. of persons	No. of samples	Salmonella			Shigella		
			Persons	Samples	Per cent. persons	Persons	Samples	Per cent. persons
László Hospital	495	1064	45	160	9.2	3	3	0.6
Semmelweis Hospital	120	203	1	1	0.8	0	0	0
Patients, Százhalombatta	249	283	7	10	2.8	3	3	1.2
Contacts, Százhalombatta	625	676	7	7	1.1	0	0	0
Healthy individuals	185	185	0	0	0	0	0	0
Total	1674	2411	60	178	3.6	6	6	0.4

* O26:K60; O55:K59; O86:K61; O111:K58; O112:K66;
O119:K69; O125:K70; O126:K71; O127:K63; O128:K67

between November, 1973, and March, 1975. Salmonellae, shigellae and enteropathogenic *E. coli* occurred in 13.8% of the patients, whereas the incidence of facultatively enteropathogenic *E. coli* serogroups was 33.1%. In 42 infants (18% of the positive cases) two or more serogroups were present; in further 42 infants the facultatively enteropathogenic *E. coli* occurred together with pathogenic enterobacteria; from 9 infants (4%) two or more pathogenic organisms were isolated. One infant harboured 7 different, 8 infants 4 or 5 different agents. In view of the frequent incidence of two or more organisms, in Table I only the results obtained at the first bacteriological examination are included. If all repeated examinations are considered, the total number of infants positive for enteric pathogens was 86 (17.3%), whereas those harbouring facultatively enteropathogenic *E. coli* numbered 194 (39%).

Most patients of this group were under 1 year of age. The frequency of facultatively enteropathogenic *E. coli* strains tended to decrease with the patients' age.

Table II shows the distribution of facultatively enteropathogenic *E. coli* isolations according to serogroups. Strains O13, O28a,c, O58, O112a,b, O135, O136 and O144 were not encountered in any of the persons examined; serogroup O73 was isolated only at a repeated examination and was omitted from Table II.

In László Hospital, 18 out of the 31 *E. coli* serogroups were met with. Two of them (O73, O112a,c) were detected at repeated examinations. The most frequent serogroups (O4, O18a,c, O25, O75 and O78) comprised 83% of the isolations.

Table III shows the incidence of facultatively enteropathogenic *E. coli* serogroups in the throat specimens. A total of 84 out of 396 infants showed the presence of the serogroups searched for. In 43 cases the same serogroup was isolated from faeces and throat swabs.

facultatively pathogenic E. coli in different groups of persons

<i>E. coli</i> O124:K72			<i>E. coli</i> associated with infantile enteritis*			Facultatively enteropathogenic <i>E. coli</i>		
Persons	Samples	Per cent, persons	Persons	Samples	Per cent, persons	Persons	Samples	Per cent, persons
0	2	0	20	27	4.0	164	389	33.1
0	0	0	3	3	2.5	32	46	26.7
1	2	0.4	7	8	2.8	45	50	18.1
0	0	0	6	6	1.0	93	98	14.9
0	0	0	0	0	0	28	28	15.1
1	4	0.1	36	44	2.2	362	611	21.6

Table II
Distribution of E. coli serogroups in different groups of persons

Serogroups	László Hospital, 495 persons		Simmelweis Hospital, 120 persons,		Patients, Százhalombatta, 249 persons		Contacts, Százhalombatta, 625 persons		Healthy individuals, 185 persons		Total, 1674 persons	
	No.	per cent	No.	per cent	No.	per cent	No.	per cent	No.	per cent	No.	per cent
O4	46	9.29	6	5.00	9	3.61	10	1.60	7	3.78	78	4.66
O18a,b	1	0.20	2	1.67	0	0	4	0.64	0	0	7	0.42
O18a,c	49	9.90	7	5.83	12	4.82	20	3.20	9	4.86	97	5.79
O19	3	0.61	0	0	1	0.40	6	0.96	0	0	10	0.60
O20	8	1.62	5	4.17	3	1.20	5	0.80	2	1.08	23	1.37
O25	11	2.22	2	1.67	4	1.61	16	2.56	0	0	33	1.97
O28a,b	0	0	0	0	0	0	2	0.32	0	0	2	0.12
O32	0	0	1	0.83	0	0	0	0	0	0	1	0.06
O51	4	0.81	1	0.83	0	0	1	0.16	1	0.54	7	0.42
O53	0	0	0	0	0	0	2	0.32	0	0	2	0.12
O75	20	4.04	3	2.50	8	3.21	7	1.12	2	1.08	40	2.39
O78	10	2.02	0	0	3	1.20	3	0.48	3	1.62	19	1.14
O79	1	0.20	0	0	0	0	2	0.32	0	0	3	0.18
O105	0	0	0	0	0	0	1	0.16	0	0	1	0.06
O112a,c	0	0	1	0.83	0	0	0	0	1	0.54	2	0.12
O114	4	0.81	3	2.50	0	0	0	0	1	0.54	8	0.48
O115	0	0	0	0	1	0.40	6	0.96	1	0.54	8	0.48
O117	1	0.20	1	0.83	3	1.20	1	0.16	1	0.54	7	0.42
O129	0	0	0	0	0	0	3	0.48	0	0	3	0.18
O142	1	0.20	0	0	0	0	1	0.16	0	0	2	0.12
O143	3	0.61	0	0	1	0.40	3	0.48	0	0	7	0.42
O145	1	0.20	0	0	0	0	0	0	0	0	1	0.06
O148	1	0.20	0	0	0	0	0	0	0	0	1	0.06
Total	164	33.13	32	26.66	45	18.05	93	14.88	28	15.12	362	21.64

Antibody titre of paired sera was determined against *E. coli* serogroups isolated from the faeces or throat of the infants. In a total of 248 sera there was a definite rise in antibody titre against 7 different serogroups of facultatively enteropathogenic *E. coli* and against two different *Salmonella* serotypes and *E. coli* O124.

Data for selected cases are shown in Table IV. There was no distinct correlation between antibody titres and the onset of the disease or the interval between two or more blood samplings. For example, in patient R. P., the titre against *E. coli* O4 increased to 1:1024 as early as on the third day. High

Table III
Distribution of E. coli serogroups isolated from throat swabs

Serogroup	László Hospital, 396 patients		Simmelweis Hospital, 118 patients	
	persons	per cent	persons	per cent
O4	36	9.1	5	4.2
O18a,c	36	9.1	5	4.2
O19	0	0	1	0.8
O75	9	2.3	0	0
O78	2	0.5	0	0
O114	1	0.2	0	0
Total	84	21.2	11	9.2

antibody titre to *E. coli* O75 appeared only after 33 days in infant T. A., whereas to *S. infantis* excreted throughout the disease, there was only an insignificant rise. In certain cases, the difference in titre between paired sera was considerable indicating an aetiological relationship (e.g. in infant J. F. excreting serogroup O18a,c). In patient T. Gy., who harboured serogroups O4, O20 and O78, a significant rise was shown only for antigen O78.

Isolations of facultatively enteropathogenic *E. coli* strains exhibited no seasonal peaks characteristic of many enteral infections.

The clinical course of the illness believed to be associated with facultatively enteropathogenic *E. coli* was analysed in 148 cases. Half of them was diagnosed as dysentery, coli enteritis or salmonellosis, but the corresponding agents were isolated only in 7%. The course of the disease was usually mild; severe enteritis was recorded only in 10%. As expected, there was no correlation between *E. coli* serogroup and clinical diagnosis. The faeces contained blood in $\frac{1}{3}$ and mucus in $\frac{1}{3}$ of the patients; normal stools were observed in $\frac{1}{5}$ of the cases (Table V). Complications (bronchitis, otitis, tonsillitis and pneumonia) appeared in 80% of the patients, respiratory symptoms predominated in 33%.

Childrens' ward, Semmelweis Hospital. Between March, 1973 and March, 1974, a total of 120 children was examined for facultatively pathogenic *E. coli* (Table I). The organisms were isolated in 26.7% from the faeces of patients without enteral symptoms (follicular tonsillitis, otitis, bronchitis, pneumonia and other acute respiratory infections). Simultaneous occurrence of two or more different serogroups in the same patient was infrequent (3%), and facultatively enteropathogenic *E. coli* serogroups were not isolated together with

Table IV
Antibody titres against enterobacteria in sera of individual patients

Patient	Age, months	Diagnosis	Bacteriological finding	Positive bacteriological result, days after admission	Blood sampling, days after admission	Reciprocal antibody titre			
						I	II	III	IV
T. Gy.	4	Dysentery	<i>E. coli</i> O4 <i>E. coli</i> O20 <i>E. coli</i> O78	2, 3, 5, 12 3, 5, 12 12	12, 16	4 4 4	4 4 128		
R. P.	18	Dysentery Bronchitis	<i>S. flexneri</i> <i>E. coli</i> O4	3 9, 10	3	32 1024			
F. B.	9	Coli enteritis Salmonellosis Pyuria	<i>E. coli</i> O26:B6	18		16	32		
			<i>E. coli</i> O125:B15	50		8	16		
			<i>E. coli</i> O18a,c	50 64		4	4		
			<i>E. coli</i> O75	50	19, 30	16	16		
			<i>E. coli</i> O78	30, 37		16	16		
			<i>E. coli</i> O124:B17	26, 27		64	128		
V. M.	11	Salmonellosis Bronchitis Sinusitis	<i>S. enteritidis</i>	26, 27, 68		8	8		
			<i>E. coli</i> O4	21, 26, 37		16	8		
			<i>E. coli</i> O25	23	30, 37	64	128		
V. G.	8	Salmonellosis Tonsillitis	<i>S. cubana</i>	26		32	16		
			<i>S. typhi-murium</i>	3, 5, 11, 13, 15, 27		512			
			<i>E. coli</i> O4	11, 13, 30, 43, 47, 50	44	4			
			<i>E. coli</i> O73	44		4			
F. G.	10	Enteritis Anaemia	<i>E. coli</i> O125:B15	23, 27, 30		16			
			<i>E. coli</i> O75 (throat)	2	2	128			
Ö. M.	4	Dysentery Bronchopneumonia	<i>E. coli</i> O75	12	1	128			
Sz. R.	14	Otitis Tonsillitis Enteritis	<i>E. coli</i> O75 (throat)	4, 5, 13	4, 13	64	128		

5	K. A.	12	Virus infection	<i>E. coli</i> O18a,c	5, 12	5, 16	128	128		
	H. M.	8	Enteritis Anaemia Marasmus	<i>E. coli</i> O117	7, 18	4, 18	32	256		
	Sz. Z.	6	Dysentery Coli enteritis	<i>S. typhi-murium</i>	4-51		4	64	32	8
				<i>E. coli</i> O18a,c	16, 32, 55, 63		4	4	4	4
				<i>E. coli</i> O78	21, 23	6, 29, 42, 48	4	32	8	8
				<i>E. coli</i> O75	30		16	32	16	16
				<i>E. coli</i> O4	34		4	4	4	4
	J. F.	7	Salmonellosis							
			Infantile atrophy	<i>E. coli</i> O18a,c	33-46		8	128	32	
			Anaemia	<i>E. coli</i> O4 (throat)	13	33, 46, 50	4	4	4	
	B. A.	6	Otitis							
			Bronchopneumonia							
Acta Microbiologica Academiae Scientiarum Hungaricae 23, 1976	B. A.	6	Salmonellosis	<i>S. panama</i>	1-28	1, 14	4	64		
			Marasmus	<i>E. coli</i> O18a,c	1-40		4	4		
	K. R.	3	Salmonellosis	<i>S. saint-paul</i>	4-21		4	4		
			Anaemia	<i>E. coli</i> O19	4	4, 14	4	64		
	T. A.	6	Bronchitis							
			Salmonellosis	<i>S. infantis</i>	3-38	2, 33	8	32		
	G. T.	4	Enteritis	<i>E. coli</i> O75	28		4	1024		
			Virus infection	<i>E. coli</i> O143	7		4	4		
			Tonsillitis	<i>E. coli</i> O18a,c	7	2, 33	8	64		
	F. J.	3	Enteritis	<i>E. coli</i> O78	25		4	128		
				<i>E. coli</i> O75 (throat)	8	8	256			

Table V

Distribution of E. coli serogroups according to type of faeces, László Hospital

Serogroup	Bloody	Mucous	Watery	Normal	Total	
					No. of patients	per cent
O4	13	20	8	3	44	29.7
O18a,c	15	15	7	12	49	33.1
O19	1	—	—	2	3	2.0
O20	4	1	1	2	8	5.4
O25	1	5	—	4	10	6.8
O75	8	5	5	4	22	14.9
O78	2	5	—	2	9	6.1
O114	2	—	1	—	3	2.0
Total	46	51	22	29	148	100.0

enteropathogenic agents. *E. coli* serogroups were significantly more frequent in infants under one year of age than in older patients ($\chi^2 = 8.4$; $p < 1\%$).

As seen in Table II, in Semmelweis Hospital 14 *E. coli* serogroups were encountered. Three of them (O78, O145, O148) occurred together with others and were, therefore, omitted from Table II. Serogroups most frequently isolated were O4, O18a,b, O18a,c, O20, O25, O75 and O114 (90.7% of all isolates). Facultatively enteropathogenic *E. coli* occurred most frequently in the spring months.

From throat specimens (Table III) of 11 patients (9.2%) three different serogroups were isolated.

Sporadic cases of enteritis, Százhalombatta. Faecal specimens were collected between March, 1973 and March, 1975. The incidence of facultatively enteropathogenic *E. coli* was 18.1% (Table I); more than one serogroup occurred in 1.6% of the patients. There was no significant difference between patients under and above 1 year of age.

Eleven out of the 31 serogroups examined were encountered; O18a, b was isolated together with another serogroup. Serogroups O4, O18a,c, O20, O25, O75, O78 and O117 were shown in 97.6% of the positive cases (Table II). The frequency of isolation showed no seasonal change.

Environment of patients with enteritis, Százhalombatta. As seen in Table I row 4, 625 persons living in the family of patients with enteritis were examined. Facultatively enteropathogenic *E. coli* serogroups were isolated in 14.9%; as compared to their incidence in the patients, the difference was not significant. Similarly, there was no difference according to age under and above 1 year.

Table II shows that persons in the environment of the patients harboured 18 different serogroups, of which O4, O18a,c, O19, O25, O75 and O115 amounted to 70% of all isolates. Serogroups O4, O18a,c, O75 and O78 occurred significantly more frequently in patients than in persons in their environment ($\chi^2 = 10.2$, $p < 1\%$), but there was no difference according to age groups.

In view of the above findings the question arose whether *E. coli* serogroups occurred frequently in the contacts because they had acquired the organisms from the patients, or whether positive findings both in patients and in healthy persons were simply due to a wide-spread even distribution of the agent in inhabitants of the town. The records showed that two or more healthy persons carrying the same organism isolated from the patient in their environment occurred only in 9 out of 90 families. In some families the same agent was demonstrated at repeated examination and in several members; e.g. in one of them *E. coli* O18a,c was excreted by the grandparents, parents and the infant throughout the 45 days of the study.

Healthy persons. Enteropathogenic agents were not isolated (Table I). Facultatively enteropathogenic *E. coli* strains occurred in 15.1%; as compared to other groups of persons, there was no significant difference in incidence. In infants the agent occurred significantly more frequently than in persons above 1 year ($\chi^2 = 10.8$; $p = 0.1\%$). As shown in Table II, 10 out of the 31 serogroups were encountered; O4, O18a,c, O20, O75 and O78 strains amounted to 82% of all isolates. Persons harbouring the same serogroups were not connected with one another.

Discussion

During a two-year period, 2411 faecal and 514 throat specimens were examined for 31 different facultatively enteropathogenic *E. coli* serogroups. The organisms were encountered frequently and in their incidence and serogroup distribution there was no significant difference between the groups of persons examined. According to age groups no difference was shown in patients with enteritis, whereas in healthy individuals and among patients with extra-intestinal diseases facultatively enteropathogenic *E. coli* occurred significantly more frequently in infants. There was, however, no difference in this respect between infants in the various groups. *E. coli* O4 and O18a,c were the prominent agents in all groups of persons. The five most frequent serogroups (O4, O18a,c, O25, O75 and O78) made up 73.7% of all positive isolations. The incidence of these organisms showed no seasonal differences and there were no clinical symptoms associated with the presence of one peculiar serogroup.

In some families the same serogroup was isolated from the patient and from healthy individuals. The multiple occurrence of a given serogroup in one family does not necessarily indicate that the patient is responsible for

its spread; there are literary data [33] that members of the same community are frequently colonized by the same microorganisms.

The rise of antibody titre in paired sera from infants with enteritis suggested the pathogenic role of certain *E. coli* serogroups (O4, O18a,c, O19, O25, O75, O78 and O117) in a few cases. In this respect it is remarkable that in the simultaneous presence of more serogroups in the patient's faeces, the antibody titre increased only against one of the agents.

It may be concluded that "facultatively enteropathogenic" *E. coli* serogroups, which have been described in association with outbreaks of enteritis, are common inhabitants of the human intestine, and single or multiple colonization with them occurs in about the same frequency in different groups of individuals. Accordingly, identification of these organisms on the basis of O antigens, unless other data support the finding, gives no information concerning their aetiological role.

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TAXONOMY OF PRIMYCIN PRODUCING ACTINOMYCETES

I. DESCRIPTION OF THE TYPE STRAIN OF THERMOMONOSPORA GALERIENSIS

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Summary. Strain FBUA 1274 (= strain 28/650707, Hungarian National Collection of Medical Bacteria, National Institute of Public Health, Budapest) is designated the type strain of *Thermomonospora galeriensis* (Vályi-Nagy *et al.*) comb. nov., and the morphological characteristics of this strain are described.

In 1949, VÁLYI-NAGY and his co-workers isolated a strain of *Streptomyces* (*Actinomyces* sensu Krasil'nikov) which proved to be able to inhibit the growth of Gram-positive bacteria and mycobacteria. From the liquid culture of this organism a new antibiotic, called primycin, was isolated [1] and the producing organism has been designated as a new species in the genus *Streptomyces*: *Streptomyces* (*Actinomyces*) *primycini* Vályi-Nagy *et al.* [2]. *Streptomyces* (*Actinomyces*) *primycini* was thoroughly studied by SZABÓ and PREOBRAZHENSKAYA in 1962 [3] who found that the organism was sterile: it did not produce sporophores and spores. According to their opinion, *A. primycini* n. sp. belongs to the heterogeneous group of *Actinomyces albus sterilis*. In 1965, VÁLYI-NAGY *et al.* isolated from the intestinal canal of the wax moth (*Galleria melonella*) a new type of primycin-producing organism [4]. They found that this new organism was not identical with *S. primycini* and termed it *Thermopolyspora galeriensis* [4] and later *Micromonospora galeriensis* [5] on account of its origin and morphological and physiological properties. The studied strains of this new species proved to be capable of producing primycin in industrially utilizable quantities. Nevertheless, a detailed description of *M. galeriensis* was up to now lacking and type strains have not been designated either for *S. primycini* or for *M. galeriensis*.

The purpose of the present report was to designate a type strain of *M. galeriensis* and to give a description of it.

Materials and methods

Bacterial strain. The *T. galeriensis* strain was isolated by VÁLYI-NAGY *et al.* [4] from the intestinal canal of the wax moth (*Galleria melonella*) and deposited as 28/650707 under the name *Thermopolyspora galeriensis* at the Hungarian National Collection of Medical Bacteria,

National Institute of Public Health, Budapest. A descendant of this strain — which is designated the type strain of *M. galeriensis* — was deposited in the culture collection of the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest, as *M. galeriensis* FBUA 1274. This strain was maintained on yeast extract-soluble starch agar at 4°C.

Media and methods for characterization. Spore shape and surface ornamentation were detected by direct electron microscopic observations of unfixed and unstained whole specimens harvested from the surface growth of the culture on agar surface. The anatomy of hyphal filaments and spores was studied by thin sectioning. Yeast-malt agar, glycerol-asparagine agar, tyrosine agar, peptone-yeast-iron agar [6], peptone-glycerol agar, starch agar and nutrient broth were used for cultural and morphological observations. The ability to utilize various carbon compounds was determined in yeast extract agar proposed by LUEDEMANN [7]: 0.5% yeast extract, 0.1% CaCO_3 , and 1.5% agar. Carbon compounds (except for inulin, dulcitol and inositol) were dissolved in distilled water and sterilized by filtration through Seitz EK filters and added to the heat-sterilized basal medium at 1.0% concentration, except for glycerol, which was added at 2.0%. The ability to utilize various amino acids as sole source of nitrogen was determined in Pridham and Gottlieb's synthetic agar medium [6]. Starch hydrolysis was determined by using a medium of the following composition: 1.0% glucose, 1.2% peptone, 0.6% meat extract, 1.0% starch, 1.8% agar, pH 7.2. Temperature range of growth was observed on a medium containing 1.0% yeast extract, 2.0% soluble starch and 1.5% agar. Nitrate reduction was tested partly in Difco nitrate broth, partly in a nitrate broth consisting of 0.5% yeast extract, 1.0% glucose, 0.5% KNO_3 , 0.1% CaCO_3 , and 1000 ml distilled water [7]. Sodium chloride tolerance was determined in a basal medium containing 1.0% yeast extract, 2.0% soluble starch and 1.5% agar [7]. Sensitivity to antibiotics was determined by the use of sensitivity disks manufactured by the Institute for Serobacteriological Production and Research "Human", Budapest.

Results

Description of the type strain (HNCMB 28/650707 = FBUA 1274) of *Thermomonospora galeriensis* (Vályi-Nagy et al.) comb. nov.

Objective synonyms: *Thermopolyspora galeriensis* Vályi-Nagy, Kulcsár, Szilágyi, Valu, Magyar, Horváth and Hegyaljai-Kiss, 1966; *Micropolyspora galeriensis* Vályi-Nagy and Kulcsár, 1969.

Morphology. The hyphal filaments, which rarely fragment, are 0.4 to 0.7 μm in diameter. Sporulation is, generally, scant. The surface of the colonies may be covered with a very thin greyish veil resembling an aerial mycelium. This greyish bloom often contains spore-bearing hyphae. Single spores are formed only on aerial mycelium. Sporophore branching is monopodial. The spores are spherical and oval, smooth or moderately warty-walled, and are 0.5 to 1.4 μm in diameter (Figs 1-3).

Cultural characteristics. No growth on glycerol-glycine agar and on sucrose-nitrate agar. Glucose-asparagine agar: colonies small, 1-2 mm in diameter, developing slowly. They are pale yellow in colour. No pigment is found in the medium. Growth on yeast-malt agar is abundant. The colonies are brownish-yellow to pale yellow in colour. The medium is coloured slightly brown. Starch agar: colonies small, 1-2 mm in diameter, developing slowly; pale green to green in colour. No soluble pigment. Glycerol-asparagine agar: colonies small, 1-2 mm in diameter, developing slowly. Substrate mycelium pale yellow to grayish-yellow. No soluble pigment. Abundant growth on peptone-glycerol agar. The well-developed colonies are wrinkled and pale



Fig. 1. Whole-mount silhouette of a hypha bearing sessile, moderately warty-walled spores. Scale marker, 1.0 μ m

yellowish-brown in colour. No soluble pigment. Growth on peptone-yeast-iron agar is abundant. Colonies are well-developed and wrinkled. The colonies are pale or grayish-yellow to brownish-yellow in colour. Growth on tyrosine agar is fair. The colour of the colonies is grayish-yellow to pale yellow or colourless. No melanoid pigment formation in peptone-yeast-iron agar and tyrosine agar.

Physiological characteristics. Nitrate and ammonium salts do not serve as sole sources of nitrogen. No growth in the presence of amino acids as sole sources of nitrogen. Simmons' citrate: no growth. Carbon utilization: D-xylose, D-glucose, D-levulose, sucrose, trehalose, and D-mannitol are utilized for growth. Trace of growth with L-arabinose, lactose, melibiose, melezitose, raffinose, inulin, dulcitol, i-inositol, and D-sorbitol. Fair growth (slightly better than the negative control) with L-rhamnose, glycerol and salicin.

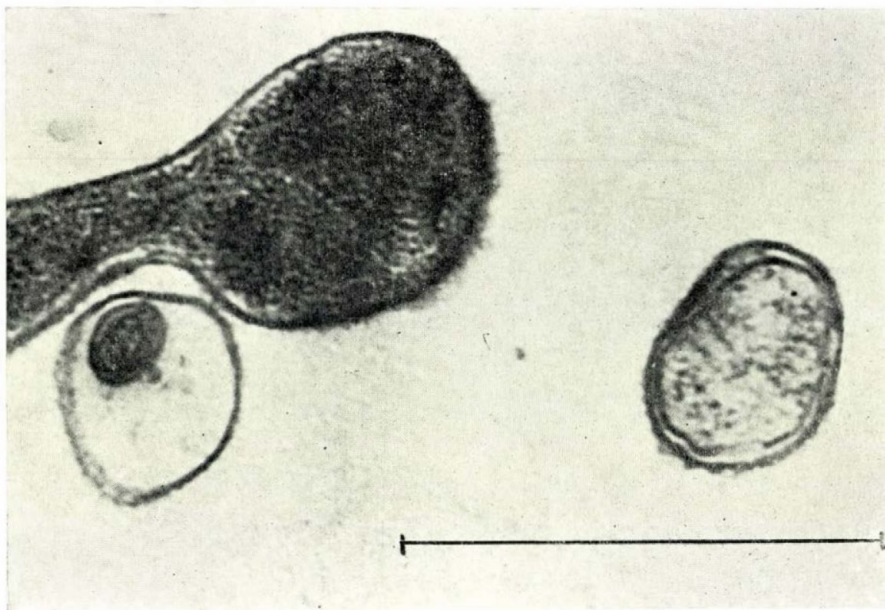


Fig. 2. Longitudinal thin section of a spore bud. Scale marker, 1.0 μm

Gelatin, 30 days 26 °C, stab culture: proteolytic action weak. Growth very slow. Very slow liquefaction. No pigment in gelatin. Hydrogen sulphide produced (weakly) from peptone. Urease test on Christensen's agar medium: weakly positive. Hydrolysis of Tween 80 as tested by Sierra's method: positive. Starch hydrolysis: positive. Cellulose strips are not attacked. Blood agar: haemolysis weak. Casein hydrolysis negative. Temperature relations: growth at 20 °C and 52 °C but not at 54 °C. Does not survive 90 °C for 5 min. Nitrate reduced to nitrite. Good growth on solid medium with 0 to 9.0% NaCl. Acetyl-methylcarbinol not produced. Catalase is produced. Methyl red test is negative. Indole not produced. Paraffin not utilized. Aerobic. Susceptibility to the following antibiotics was observed: Chloramphenicol (30 μg), chlortetracycline (30 μg), erythromycin (10 μg), kanamycin (30 μg), novobiocin (30 μg), oleandomycin (30 μg), oxytetracycline (30 μg), spiramycin (30 μg), streptomycin (30 μg), tetracycline (30 μg), vancomycin (50 μg). The organism was resistant to penicillin (3IU), oxacillin (10 μg), polymyxin-B (15 μg).

Discussion

M. galeriensis bears a resemblance to *Thermomonospora viridis* (Shuurmans et al.) Küster and Locci 1963. [8]. After isolation it produced a dark green endopigment on certain media. The intensity of this green pigmen-

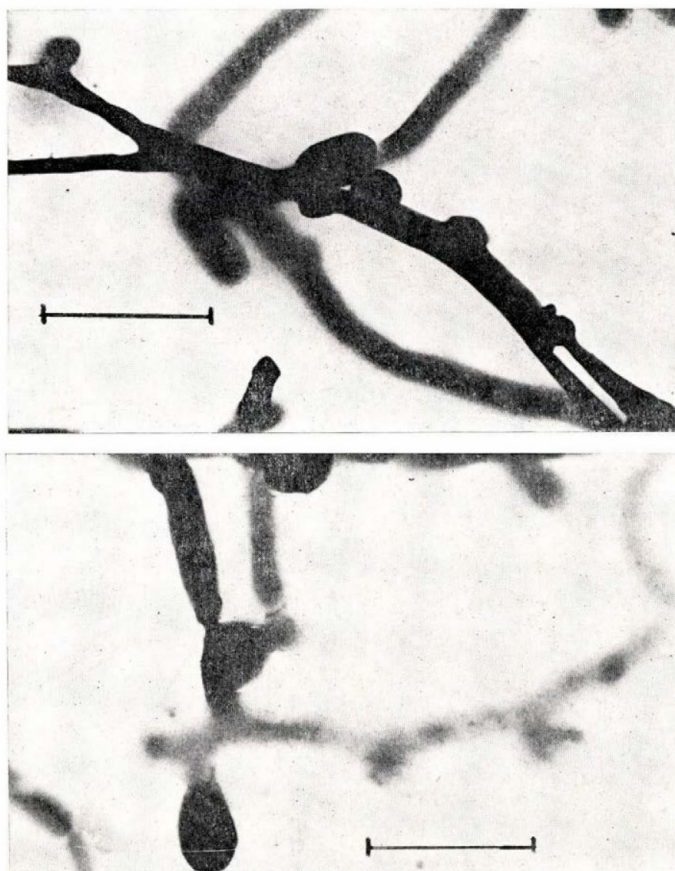


Fig. 3. Mature, smooth-walled, oval-shaped spores produced singly on lateral side branches at the surface of the colonies on yeast-malt agar. Scale markers, 2.0 μ m

tion of substrate mycelium gradually decreased during the long period of artificial cultivation.

T. galeriensis, however, does not produce green aerial mycelium and dark green soluble pigment. Besides, it grows well at 20°C and does not grow at 55°C. *T. galeriensis* seems to be a valid species within the genus *Thermomonospora* Henssen 1957. Morphologically it is only one of the many members of that range of morphological types of monosporic actinomycetes which includes the genera *Thermomonospora* and *Thermoactinomyces* overlapping their generic boundaries.

The C-source utilization patterns of *Thermomonospora* strains frequently show considerable instability or temporal variability. VÁLYI-NAGY *et al.* [4] found that strain 28/650707 of *M. galeriensis* was able to utilize rhamnose and glycerol. Using yeast-extract agar as basal medium we found that in the

presence of these C-sources the growth of strain 28/650707 was only slightly better than the negative control. Repeated trials gave similar results.

In the second part of our studies on the primycin producing organisms we shall present the results of a simultaneous comparison between the type culture of *T. galeriensis* and authentic strains of *Streptomyces (Actinomyces) primycini* and *Thermonospora viridis*.

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REMARQUES SUR L'ÉVOLUTION DE LA PAROI CELLULAIRE AU COURS DE LA SPORULATION DE LA LEVURE *SACCHAROMYCES CEREVISIAE* HANSEN

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D. BOUDIER, M. ROGER, F. VEZINHET, M. BASTIDE, J. B. BELIN and P. GALZY (*Research Laboratory, Institute of Genetics, I.N.R.A.-E.N.S.A. and Laboratory of Immunology, Virology and Parasitology, Faculty of Pharmacy, Montpellier*): **Remarks on the modifications of cell wall during the sporulation of *Saccharomyces cerevisiae* Hansen.** Evolution of cell wall during sporulation was studied by means of scanning electron microscopy and by immunological techniques. Experiments were done simultaneously with a strain a/α able to sporulate and a strain α/α unable to sporulate. Under such conditions it was possible to clarify whether the changes observed were related to the sporulation or to the culture conditions. Cell wall structure modifications during sporulation were not obvious morphologically but have been revealed by immunological methods. During vegetative growth, antigenic sites of strains a/α and α/α were different. During incubation in the sporulation medium, antigenic structure of the cell wall was modified. Some antigenic sites seem to be specific of sporulation.

Résumé. L'étude de l'évolution de la paroi cellulaire au cours de la sporulation a été faite par microscopie électronique à balayage et par des techniques immunologiques. L'expérimentation faite en parallèle sur une souche a/α capable de sporuler et sur une souche α/α incapable de sporuler a permis de déterminer si les modifications observées sont dues à la sporulation même ou aux conditions particulières de milieu. Les modifications de la structure de la paroi des cellules au cours de la sporulation ne se manifestent guère au niveau morphologique mais elles ont pu être mises en évidence par les méthodes immunologiques. En croissance végétative, les souches a/α et α/α diffèrent par quelques sites antigéniques. L'immersion des cellules dans le milieu de sporulation s'accompagne d'un remaniement de la structure antigénique de la paroi. Certains sites semblent spécifiques de la sporulation.

La sporulation de la levure *Saccharomyces cerevisiae* est un phénomène de morphogénèse complexe. La paroi cellulaire doit être le siège de modifications importantes. En effet, sa fonction se modifie considérablement au cours de ce processus. Cette structure cellulaire qui a un rôle biologique important chez la cellule végétative devient progressivement une simple enveloppe de protection des spores.

La paroi de la cellule végétative a fait l'objet de nombreuses études. Plusieurs auteurs [1-6] en ont déterminé la composition et la structure.

Par contre, il existe peu de données dans la bibliographie concernant l'évolution de la composition et de la structure de la paroi au cours de la sporulation: SNIDER et MILLER [7] par des méthodes immunologiques n'ont

pas observé de différence entre la paroi des cellules végétatives et celle des asques. Par contre, ils ont constaté des différences pour la paroi des spores. D'autre part, la paroi des asques est plus sensible que celle des cellules végétatives au suc gastrique d'escargot [8]. Ce fait est en faveur d'une différence de composition chimique ou de structure entre ces deux enveloppes.

Dans ce travail, deux techniques ont été mises en œuvre pour étudier la paroi cellulaire au cours de la sporulation: la microscopie électronique à balayage et l'immunofluorescence indirecte. Les expériences ont été faites en parallèle sur une souche a/α capable de sporuler et sur une souche α/α incapable de sporuler. Cette deuxième souche sert de témoin et permet de contrôler si les modifications observées sont réellement dues à la sporulation ou à tout autre facteur indépendant de la sporulation.

Matériel et méthodes

Matériel biologique. Deux souches diploïdes de *Saccharomyces cerevisiae* Hansen ont été utilisées. Ces souches ont été isolées au laboratoire par PELLECUER [9] à partir de la souche 6153 C9 P1 (10). Elles sont isogéniques sauf pour le gène déterminant le signe. Leur génotype est le suivant:

532	a/α	ADE6	ADE3-3	URA1
		ade6	ade3-3	ura1
533	α/α	ADE6	ADE3-3	URA1
		ade6	ade3-3	ura1

Milieu de culture. Les souches ont été cultivées sur un milieu contenant de l'extrait de levure Difco 0,5% et du glucose 0,5%. Les cultures sont faites en milieu agité (80 oscillations/mn, amplitude 7,5 cm) dans des erlenmeyers remplis au 1/10 de leur volume.

Milieu de sporulation. Mélange en parties égales de tampon phosphate 66 mM, pH 7, et d'acétate de potassium 400 mM, pH 7.

Technique de microscopie électronique. L'étude en microscopie électronique à balayage a été effectuée avec des cellules de levures fixées et préparées selon la méthode de PARUCZ [11] modifiée par SMALL et MARSZALEK [12]. Les cellules sont mises en suspension dans le fixateur de PARUCZ: mélange en proportions 6/1 (v/v) d'une solution aqueuse d'acide osmique OsO_4 à 2% et d'une solution aqueuse saturée de chlorure mercurique. Après un traitement de 15 minutes à la température ambiante le fixateur est éliminé; les cellules sont lavées à l'eau distillée et vaporisées sur des disques d'aluminium préalablement refroidis par de l'azote liquide; elles sont ensuite lyophilisées et recouvertes d'un mince film d'or (800 Å d'épaisseur). Les échantillons sont examinés à l'aide d'un microscope électronique à balayage Cambridge Stereoscan (modèle SA 10, voltage d'accélération 20 kv).

Techniques immunologiques. Un lot de 4 lapins est affecté à la préparation de chaque sérum. Les injections d'antigènes (suspensions cellulaires dans de l'eau physiologique) sont faites par voie sous-cutanée 1 fois par semaine, pendant 4 mois. Le volume de l'injection et la concentration en antigènes varient de 0,1 ml à $2 \cdot 10^6$ cellules/ml la première semaine, à 0,5 ml à $10 \cdot 10^6$ cellules/ml la dernière semaine. Chaque injection est additionnée d'un volume égal d'adjuvant complet de Freund (Difco) pour améliorer la formation des anticorps.

Au terme de l'immunisation le sang est recueilli par ponction cardiaque. Une bonne exsudation de sérum est obtenue à $+4^\circ C$ pendant 20 heures. Le sérum est séparé par centrifugation 10 minutes à 10 000 tours/minute.

Trois sérums ont été préparés:

— «Sérum anti 532 — exponentielle» obtenu par injection de cellules de la souche 532 recueillies en phase exponentielle de croissance.

— «Sérum anti 533 — exponentielle» obtenu par injection de cellules de la souche 533 recueillies en phase exponentielle de croissance.

— «Sérum anti 533 — 48 h — sporulation» obtenu par injection de cellules de la souche 533 recueillies après 48 heures d'immersion dans le milieu de sporulation.

Le «sérum anti — 532 — 48 h — sporulation» n'a pas été fabriqué car à ce stade, la population cellulaire de la souche 532 est hétérogène. La culture contient des cellules en voie

de sporulation et des cellules qui ne sporuleront pas. Ces deux types de cellules semblent avoir des propriétés pariétales différentes d'après des expériences réalisées au laboratoire (résultats non publiés). En conséquence, les résultats obtenus avec un tel sérum auraient été très difficiles à analyser.

Chacun des trois sérums est adsorbé par diverses préparations cellulaires, ce qui a pour conséquence d'éliminer les anticorps correspondant aux antigènes communs aux deux suspensions cellulaires concernées.

- cellules de la souche 532 en phase exponentielle de croissance
- cellules de la souche 533 en phase exponentielle de croissance
- cellules de la souche 532 après 48 heures d'incubation dans le milieu de sporulation
- cellules de la souche 533 après 48 heures d'incubation dans le milieu de sporulation.

Les réactions immunologiques ont été effectuées par la méthode de fluorescence indirecte de COONS selon BASTIDE *et al.* [13]. Ces réactions mettent en présence les anticorps des sérums adsorbés, des antiglobulines de lapin et les antigènes suivants:

- cellules de la souche 532 en phase exponentielle de croissance
- cellules de la souche 533 en phase exponentielle de croissance
- cellules de la souche 532 après 48 heures d'immersion dans le milieu de sporulation
- cellules de la souche 533 après 48 heures d'immersion dans le milieu de sporulation
- cellules de la souche 532 après 72 heures d'immersion dans le milieu de sporulation
- cellules de la souche 533 après 72 heures d'immersion dans le milieu de sporulation.

Les antiglobulines de lapin, marquées avec un fluorochrome (isothiocyanate de fluoresceïne) sont commercialisées par B. D. Merieux. Au microscope les parois cellulaires qui ont fixé le complexe d'anticorps marqué apparaissent fluorescentes.

Résultats

I. *Étude par microscopie à balayage.* Les cellules végétatives des deux souches, prélevées en phase exponentielle de croissance sur le milieu de culture, sont immergées après un lavage à l'eau physiologique dans le milieu de sporulation. Au bout d'un temps variable de contact avec le milieu de sporulation, une fraction cellulaire est préparée pour l'étude au microscope à balayage.

— Les cellules en croissance végétative (photos 1 et 2) se multiplient par bourgeonnement. La scission des cellules se traduit à la surface cellulaire par la présence de cicatrices de deux types: les cicatrices de bourgeonnement, visibles sur les cellules mères, les cicatrices de naissance des cellules filles [14–16]. Les cellules mères, âgées, portent de nombreuses cicatrices et ont une paroi plissée. Les cellules filles présentent un aspect plus lisse et une forme plus allongée.

— Pendant les six premières heures de contact avec le milieu de sporulation (photos 3 et 4) de nombreuses cellules augmentent de volume, prennent une forme sphérique et un aspect lisse.

— Au bout de 18 heures (photos 5 et 6) l'augmentation de taille des cellules est nette. Les cellules ont pris une forme globuleuse et un aspect plissé. Jusqu'à ce stade, aucune différence morphologique n'a pu être mise en évidence entre les deux diploïdes.

— Après 30 heures d'immersion dans le milieu de sporulation, les spores apparaissent dans les cellules de la souche 532. L'aspect plissé se maintient durant tout le processus de sporulation chez la souche 532 et se retrouve chez la souche 533.



Photo 1. Souche 532. Phase exponentielle.
×5000

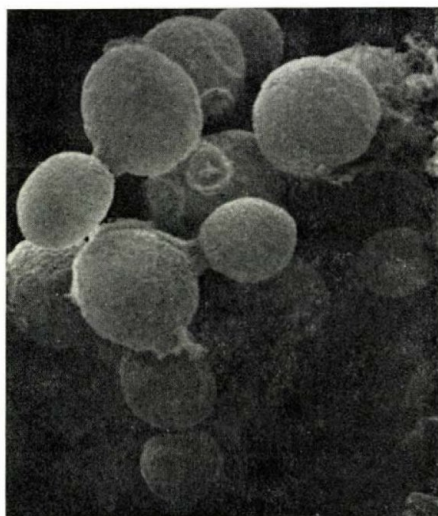


Photo 2. Souche 533. Phase exponentielle.
×5000

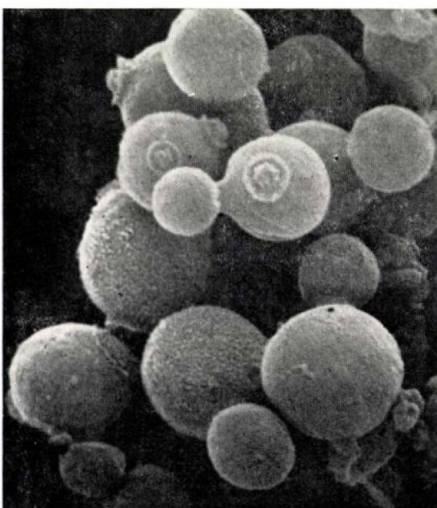


Photo 3. Souche 532 après 6 hr d'immersion
dans le milieu de sporulation. ×5000

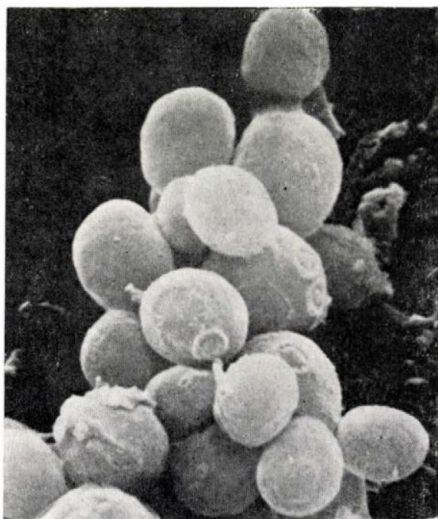


Photo 4. Souche 533 après 6 hr d'immersion
dans le milieu de sporulation. ×5000

— Après 72 heures de contact avec l'acétate, la sporulation peut être considérée comme achevée. Les spores exercent une tension sur la paroi de l'asque de la souche qui a sporulé (photo 7). La photo 8 montre l'aspect des cellules de la souche 533 au même stade.

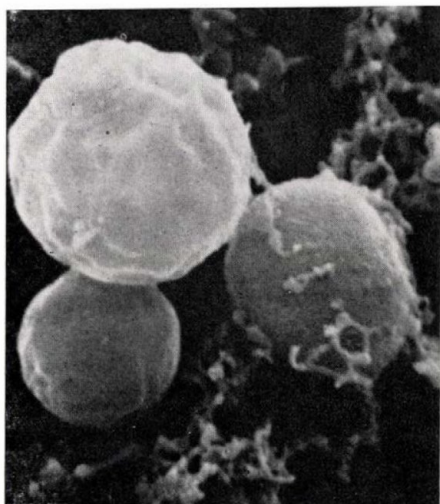


Photo 5. Souche 532 après 18 hr d'immersion dans le milieu de sporulation. $\times 10000$

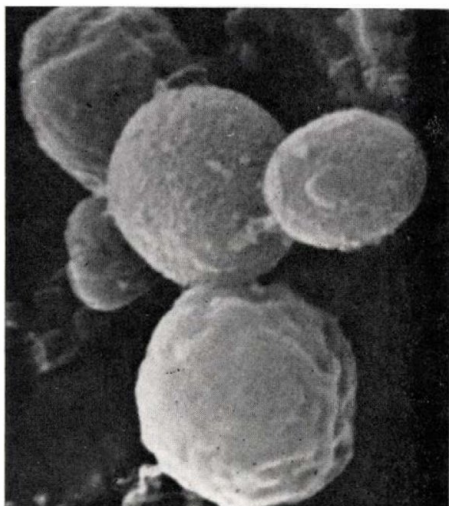


Photo 6. Souche 533 après 18 hr d'immersion dans le milieu de sporulation. $\times 10\ 000$

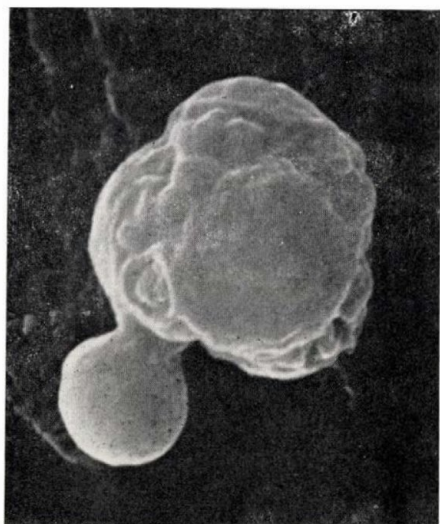


Photo 7. Souche 532 après 72 hr d'immersion dans le milieu de sporulation. $\times 10\ 000$

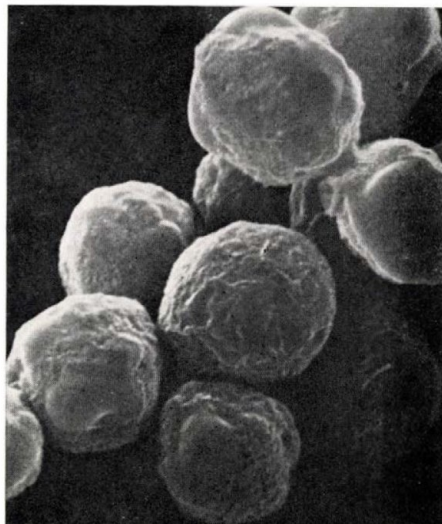


Photo 8. Souche 533 après 72 hr d'immersion dans le milieu de sporulation. $\times 5000$

Cette étude de l'aspect extérieur des cellules au cours de l'immersion dans un milieu de sporulation, n'a pas permis d'observer de différences entre les deux souches jusqu'à l'apparition des asques chez la souche 532. L'incubation dans le milieu acétate ne provoque pas de profondes modifications de l'aspect

pariétal des cellules. Le gonflement des cellules lors des premières heures d'immersion dans le milieu de sporulation est à rapprocher de l'activité métabolique intense à ce moment là, activité qui entraîne une accumulation de lipides et de glucides dans les cellules des deux souches [17]. Chez les cellules de la souche 532, à la fin du processus de sporulation, les spores exercent une tension au niveau de la paroi. La surface des asques ne présente pas d'ornementations particulières et n'a pas un aspect fondamentalement différent de celui des cellules non sporulées.

II. *Études immunologiques.* L'étude en microscopie électronique des diploïdes 532 et 533 révèle une grande similitude morphologique de la paroi de ces deux souches. Ce résultat paraît logique étant donné que ces deux souches ne diffèrent que par un gène. Pour mettre en évidence une différence au niveau de la paroi, il faut donc utiliser une méthode d'investigation beaucoup plus fine. Dans ce contexte, la technique d'immunofluorescence indirecte semble convenir.

Les résultats obtenus sont groupés dans le tableau 1. Les chiffres indiquent l'intensité de la fluorescence de la paroi, évaluée de 0 (fluorescence nulle) à 5 (fluorescence maximale). Les cases de ce tableau sont affectées d'un numéro (en haut, à gauche) pour faciliter l'exposé des résultats.

A) *Mise en évidence d'une différence antigénique entre les parois des deux diploïdes en phase exponentielle.*

Le sérum anti — 532 — exponentielle, adsorbé sur les cellules de la souche 533 en croissance exponentielle donne une réaction positive avec les cellules de la souche 532 en croissance exponentielle (case 1). De même, le sérum anti — 533 — exponentielle, adsorbé sur les cellules de la souche 532 en croissance exponentielle, donne une réaction positive avec les cellules de la souche 533 en croissance exponentielle (case 20).

En phase exponentielle de croissance, les souches 532 et 533 présentent des sites antigéniques différents.

B) *Mise en évidence d'une différence antigénique entre les parois des deux diploïdes après 48 heures d'immersion dans le milieu de sporulation.*

Le résultat de la case 52 montre qu'après 48 heures d'immersion dans le milieu de sporulation, la souche 533 possède des sites antigéniques que la souche 532 au même stade ne possède pas. La réciproque n'a pu être mise en évidence de façon directe puisque le « sérum anti — 532 — 48 h — sporulation » n'a pas été préparé. Toutefois les résultats des cases 27 et 15 permettent d'affirmer qu'il n'y a pas identité entre les souches 532 et 533 après 48 heures d'incubation dans le milieu de sporulation.

Le fait que les résultats des cases 19 à 24 d'une part et des cases 49 à 54 d'autre part ne sont pas identiques, montre que les différences entre les souches en phase exponentielle sont nettement plus importantes que celles qui les distinguent après immersion dans le milieu de sporulation.

Tableau 1
Résultats des réactions sérologiques

Sérums	Cellules adsorbantes	Antigènes = suspensions cellulaires					
		Cellules en phase exponentielle de croissance		Cellules après 48 h d'immersion dans le milieu de sporulation		Cellules après 72 h d'immersion dans le milieu de sporulation	
		532	533	532	533	532	533
Sérum anti 532 exponentielle	533 en exponentielle	1 0,5	2 0	3 0,5	4 0	5 0	6 0,5
	532 après 48 hr d'immersion dans le milieu de sporulation	7 0	8 0	9 0	10 0	11 0	12 0
	533 après 48 hr d'immersion dans le milieu de sporulation	13 0	14 0	15 0,5	16 0	17 0	18 0
	532 en exponentielle	19 0	20 1	21 1	22 1	23 0,5	24 0,5
	533 après 48 hr d'immersion dans le milieu de sporulation	25 0,5	26 0	27 0,5	28 0	29 0	30 0
	532 après 48 hr d'immersion dans le milieu de sporulation	31 0	32 0	33 0	34 0	35 0	36 0
Sérum anti 533 exponentielle	533 en exponentielle	37 0,5	38 0	39 0	40 0,5	41 0,5	42 0
	532 en exponentielle	43 0	44 0	45 0	46 1	47 0	48 0
	532 après 48 hr d'immersion dans le milieu de sporulation	49 0	50 0	51 0	52 0,5	53 0	54 0
Sérum anti 533 48 heures sporulation	533 en exponentielle	37 0,5	38 0	39 0	40 0,5	41 0,5	42 0
	532 en exponentielle	43 0	44 0	45 0	46 1	47 0	48 0
	532 après 48 hr d'immersion dans le milieu de sporulation	49 0	50 0	51 0	52 0,5	53 0	54 0
	533 en exponentielle	37 0,5	38 0	39 0	40 0,5	41 0,5	42 0
	532 en exponentielle	43 0	44 0	45 0	46 1	47 0	48 0
	532 après 48 hr d'immersion dans le milieu de sporulation	49 0	50 0	51 0	52 0,5	53 0	54 0

C) *Evolution des propriétés antigéniques de la paroi au cours de l'immersion dans le milieu de sporulation.*

1°) Cas de la souche 532

— La case 3 montre que la paroi des cellules de la souche 532 conserve au moins en partie ses sites antigéniques spécifiques au cours des 48 premières heures de la sporulation. Ce résultat est confirmé par la réaction de la case 15.

— Aucune réaction n'a montré la perte de sites antigéniques dans la paroi de la souche 532, pendant la sporulation (cf résultats des cases 7, 8, 9, 10, 11, 12).

— La réaction de la case 21 montre que la souche 532 acquiert des sites antigéniques nouveaux pendant le contact avec l'acétate, sites déjà présents dans les cellules de la souche 533 en croissance exponentielle. Ce phénomène atteint son maximum au bout de 48 heures; les dernières heures de la sporulation semblent s'accompagner d'une perte en sites antigéniques (case 23).

Au cours de la sporulation, la souche 532 conserve des sites antigéniques pariétaux qui lui sont propres; elle ne subit pas de pertes et au contraire s'enrichit en sites nouveaux.

2°) Cas de la souche 533

— Certains caractères antigéniques spécifiques de la souche 533 se maintiennent au cours de l'incubation dans le milieu de sporulation (case 22). Cependant, après 48 heures d'immersion dans l'acétate, la souche 533 s'appauvrit en un certain nombre de sites antigéniques (cases 25 et 27).

— L'incubation dans le milieu de sporulation entraîne l'acquisition de sites antigéniques nouveaux (case 40).

Au cours de l'immersion dans le milieu de sporulation, la souche 533 conserve quelques uns des sites antigéniques qui, en phase exponentielle, la distinguent de la souche 532. Elle subit la perte de quelques sites et s'enrichit en d'autres sites. Le maximum d'antigénicité semble avoir lieu à 48 heures de contact avec l'acétate.

Discussion

En phase exponentielle de croissance, l'aspect extérieur des cellules des deux souches 532 et 533 est identique. Toutefois, l'étude immunologique a permis de mettre en évidence des différences au niveau des sites antigéniques de la paroi. Ces sites doivent être sous le contrôle génétique des gènes *a* et *z* puisque les deux souches ne diffèrent que par le locus portant ces allèles.

Au cours de l'immersion dans le milieu de sporulation, nous avons observé une augmentation du volume des cellules, de légères modifications de la surface cellulaire; ces observations sont communes aux deux souches 532 et 533 et par suite pourraient être dues plutôt aux conditions particulières de milieu (arrêt de la croissance et présence d'acétate) qu'au phénomène proprement dit de la sporulation. L'immersion des cellules dans le milieu de sporulation s'accompagne d'un remaniement de la structure antigénique qui se solde par un enrichissement en sites antigéniques. La différence d'antigénicité entre les deux souches observée en phase exponentielle de croissance se maintient au contact de l'acétate mais semble toutefois s'atténuer. Les modifications au niveau antigénique sont-elles liées à la sporulation ou aux

conditions de milieu? Le travail présenté ici ne permet pas de conclure de façon absolue. Toutefois, l'évolution des deux souches semble être un peu différente et il est possible que certains sites antigéniques soient spécifiques de la sporulation.

Les modifications de la structure de la paroi des cellules au cours de la sporulation ne se manifestent guère au niveau morphologique mais par contre elles ont pu être mises en évidence par des méthodes immunologiques.

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